Development, validation and application of an analytical method for the determination of fucose in human plasma after oral administration of fucoidan

by

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Declarations

Declaration of Originality

This thesis contains no material that has been accepted for the award of any degree or diploma in any other tertiary institution.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due reference is made in the text of the thesis.

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Statement of Ethical Conduct

The research associated with this thesis abides by the International and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All research procedures reported in the thesis were approved by the Tasmania Health and Medical Human Research Ethics Committee or the Tasmanian Social Sciences Human Research Ethics Committee.

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8 October 2015

Statement of Co-Authorship

The following people and institutions contributed to the publication or preparation of the work undertaken as part of this thesis.

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List of Abbreviation

^{1}H-NMR	^{1}H-Nuclear Magnetic Resonance
ACC	Acetyl CoA carboxylase
ACN	Acetonitrile
Anti-HIV	Anti-Human Immunodeficiency Virus
ASSS	Ammonium Sulphate saturated solution
CAD	Charged Aerosol Detector
CCl4	Carbon tetrachloride
CD4	Cluster of differentiation 4
COA	Certificate of analysis
DNA	Deoxyribonucleic acid
DOCA	Deoxycholic acids
ECOG	The Eastern Cooperative Oncology Group
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
FRAP	Ferric reducing antioxidant power
FS	Fucose solution
FV	Filter value
GI	Gastrointestinal
GIT	Gastrointestinal tract
HCT116	Human Colorectal Carcinoma
HILIC	Hydrophilic Interaction Liquid Chromatography
HMW	High Molecular Weight
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
ICH	International Conference of Harmonisation

IUPAC	International Union of Pure and Applied Chemistry
kDa	kilo-Dalton
LMW	Low Molecular Weight
LOD	Limit of Detection
LOQ	Limit of Quantification
MCF-7	Michigan Cancer Foundation-7
MiBK	Methyl isobutyl ketone
mRNA	Messenger Ribonucleic acid
MW	Molecular weight
NHMRC	the National Health and Medical Research Council
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
ODS	Octa Decyl Silane
PAGE	Polyacrylamide Gel Electrophoresis
PF	Power Function
PPARγ	Peroxisome proliferation-activated receptor γ
PPT	Protein Precipitation
RPIP-HPLC	Reversed-Phase Ion-Pairing High Performance Liquid Chromatography
RSDs	Relative Standard Deviations
RT	Reverse Transcriptase
SEC-HPLC	Size Exclusion High Performance Liquid Chromatography
SEC-HPLC-RI	Size Exclusion High Performance Liquid Chromatography with Refractometry Index detector
SNAC	Sodium N-[8-(2-Hydroxybrnzoyl)Amino] Caprylate
TBA	Tributylamine
TFA	Trifluoroacetic Acid
UFH	Unfractionated Heparin

URN	Unit Record Number
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
W	Water

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Abstract

Background:

It has been reported that fucoidan, a polysaccharide commonly extracted from brown seaweeds, possesses a wide range of biological activities. Fucoidan is composed of a highly negatively charged and polydispersed oligosaccharides having fucose as the main component. It is not possible to separate various oligosaccharides of intact fucoidan using only one method because of their high negative charge, high molecular weight and polydispersity. Fucoidan is commercially available in the oral dosage form (only available as capsules). However, its bioavailability after oral administration is currently unknown. Therefore, the objective of this study was to develop an analytical technique that can measure the concentration of fucose (the main component of intact fucoidan) after oral administration of fucoidan. The application of newly developed method was demonstrated by analysing the fucose levels in patients' plasma before and after oral administration of fucoidan.

Method:

Different types of chromatographic methods (reversed-phase chromatography, reversed-phase ion-pairing chromatography and normal phase chromatography) were tried to obtain a method that can separate fucose from other compounds present in plasma. Once this was achieved, several analytical parameters such as mobile phase composition, buffer strength, pH, and other parameters within the detector were also tested to increase the sensitivity of the method. Digestion and precipitation methods were used to breakdown fucoidan into fucose and to precipitate fucose respectively. The developed method was then validated using inter- and intra-day precision, accuracy and reproducibility. The limit of detection (LOD) and limit of quantitation (LOQ) was also calculated using the mean standard deviation obtained from three

calibration curves. The developed and validated method was then used to determine the presence of plasma fucose levels in cancer patients before and after oral administration of fucoidan. The patients were taking either Docexatel (maximum dose between 75 and 100 mg every 3 weeks) or Anastrozole (1 mg daily). A 250 mg hard-gelatine capsule containing 187.5 mg of fucoidan was orally administered four times a day for seven days. The patients' plasma samples were precipitated with acetonitrile and digested using 20% trifluoroacetic acid to depolymerise fucoidan into fucose. The amount of fucose in the plasma samples was then determined using the developed method.

Results:

It was concluded that, although this might be limited to the particularly selected chromatographic parameters (types of column, mobile phase and ion-pairing reagents), normal phase chromatography was more suitable in the analysis of fucose compared to the reversed-phase and reversed-phase ion-pairing chromatography tested in this study. The tested normal phase chromatography method performed well in retaining and resolving fucose both in standard solution and plasma samples. Fucose-containing plasma sample was resolved using Shodex Asahipak NH2P-50 4E column employed with a series of Dionex Ultimate Pump, autosampler, column compartment and a Charged Aerosol Detector (CAD). Mobile phase consisted of 5 mM ammonium acetate pH 4.75 and acetonitrile (20:80) with the flow rate of 1.0 mL/minute. Peak to concentration correlation test using 5 different concentrations of fucose solutions (5 µg/mL to 100 µg/mL) gave exponential œrrelation coefficient (R^2) of 0.9999. Fucoidan was digested using 20% trifluoroacetic acid and was found to contain 20.35%±0.87% (n=3) of fucose (w/w). Plasma protein and spiked fucoidan from 1 mL of plasma was precipitated using 4 mL of acetonitrile. Recovery of fucose in fucoidan-spiked blank plasma after precipitation was found to be more than 92% (n=2) based on the calculated fucose

concentration. Intra- and Inter-day precision, accuracy and reproducibility values were found to be less than 5% RSD. Limit of detection (LOD) and limit of quantitation (LOQ) of the developed method was calculated to be 2.28 ng and 6.92 ng, respectively. Fucose concentration in patients' plasma after the administration of fucoidan was elevated by (1.14±0.61) mg/100 mL (n=19). This suggested that the oral absorption of fucoidan was around 14.28%±7.59% (n=19).

Conclusion:

The developed method was suitable for the detection of fucose in patients treated with oral fucoidan. The result obtained in this study showed that less than 15% of orally administered fucoidan was absorbed. Nonetheless, this shows that, to some extent, fucoidan is getting absorbed following oral administration. Moreover, the proposed method can be used in the analysis of fucoidan in human plasma as an alternative option to the currently available ELISA method or those using derivatisation. It offers simplicity in the procedure and specificity in the detection of fucose, the main component of fucoidan.

Table of Contents

List of Tables

List of Figures

Chapter 1 - Introduction

1.1 Seaweeds

Seaweeds, plant-like organisms, are the enrichment to the aquatic biodiversity and have been well recognised for its usefulness for ages. History of seaweeds dates back to early sixth century where Japanese emperors utilised seaweed as a kind of tax or processed and prepared it as food supplement[1]. Moreover, in the Far East region, seaweeds were used as traditional medicine for the treatment of several ailments including goitre[2,3]. Seaweeds were also used for other economic purposes including their supply as cattle fodder[2,4].

1.2 Classes of Seaweeds

There are thousands of seaweed species and four major classes are shown in Figure 1[2,4]. This classification is based on their thallus colour or pigmentation. These include *Chlorophycaeae* (green), *Cyanophycaeae* (blue-green), *Rhodophycaeae* (red) and *Phaeophycaeae* (brown) seaweeds. Among these four classes, *Chlorophycaeae* and *Cyanophycaeae* cover both salt or fresh water species while *Rhodophycaeae* and *Phaeophycaeae* are mostly salt water species. Seaweeds are structurally as well as functionally distinct to land plants. They do not have a complete or distinctive root and leaf system but some have bladders filled with air to help them float in the water[2]. They vary in size and appearance but their common natural living habitats are rock surfaces in coastal areas.




(1) (2) (3) (4)

Figure 1.1 Pictorial representation of four major classes of seaweed: (1) Green seaweed *Cladophora rupestris*, (2) Red seaweed *Asparagopsis armata*, (3) Brown seaweed *Fucus vesiculosus*, (4) Brown seaweed *Undaria pinnatifida* [5]

The annual economic market of seaweeds in 2009 was recorded to be approximately U.S. $ 5 billion worldwide[6]. It has also been reported that a US based company alone, selling commercial product of fucoidan (polysaccharide commonly extracted from brown seaweeds) claimed as a health supplement, has gained annual income of $6 billion in 2015[7]. This shows an increasing trend in seaweeds market, particularly fucoidan. Seaweeds can offer potential resources to fulfil human need of carbon-based food since they are found in abundance in most coastal regions around the world. The trend has shifted from consuming food merely as the need to satisfy the taste-sense towards exploring both of their nutritional and medicinal value; hence seaweed can be considered as one of the valuable option. Numerous research studies have provided an insight on seaweed's chemical components and biological activities[8-13].

1.3 Components of Seaweeds

Seaweeds are known to contain multiple components such as polysaccharides, proteins, minerals and vitamins. Seaweeds are also considered as "*functional food"* because they carry components which also exert bioactive functions. For example, brown seaweeds such as *Saccharia sp.* and *Undaria sp.* contains alginic acid, an insoluble gelatinous carbohydrate, which is known to possess antibacterial, anti-hypertensive and anticancer properties[11,14]. On

the other hand, carrageenan from *Chondrus crisprus* consisting of a mixture of polysaccharides is known for its anticoagulant and antiviral properties. Alginate or alginic acid, carrageenan and agar are also commercially used as thickeners, emulsifiers, food supplement and other applications; such as the utilisation of agar as a raw material in capsule production for medical purpose and as a medium for cell cultures[15,16]. Polysaccharides, protein, minerals and vitamins are abundant in seaweeds but their composition and amount differ depending on the type of seaweed species, season of harvesting and location[16]. For example, the concentration of alginate extracted from *Costaria costata* in July is significantly higher compared to when it is extracted in April and May[17]. Also, it has been observed that calcium is produced higher by green seaweed than that by red or brown seaweeds[8]. Among several mineral components, fluoride is present in highest amount in brown seaweeds. Zinc, even though lesser in percentage, is higher than iron. The iron fraction in red seaweed is the highest while in brown seaweed it is the lowest. These mineral-rich seaweeds can be considered to be beneficial in providing mineral supplement for those suffered from mineral deficiency[8]. Seaweeds are also composed of polysaccharides, polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic bonds. Fucoidan is one of the polysaccharide components found in seaweeds.

1.4 Fucoidan

It was Kylin, a professor of Uppsala University in Sweden, who first isolated fucoidan from *Laminaria sp.* and *Fucus sp.* species of brown seaweeds in 1913 and was initially named *fucoidin* based on the source of its isolation and later to fucoidan to conform with polysaccharide nomenclature[18]. Fucoidan is highly negatively charged, sulphated polysaccharide and exist only in brown seaweeds. The structure of fucoidan differs to the other

main type of polysaccharide from other classes of seaweeds such as *Rhodophycaeae* or red seaweeds, as it contains fucose as the backbone structure instead of galactose[19].

1.4.1 Sources

Fucoidan is most commonly extracted from brown seaweed, although it can also be extracted from some marine invertebrates such as body wall of sea cucumbers or egg jelly coat of sea urchins[18,20]. It is widely spread in all species of brown seaweed but not in other classes of seaweeds such red seaweed, green seaweed and golden seaweed[18]. The chemical or structural composition of fucoidan varies according to the type of seaweed, season of harvesting, local climate and type of extraction method employed[17,19]. Some brown seaweeds species, for example *Fucus vesiculosus*, have a relatively simple composition containing only fucose and sulphate groups; while fucoidan from *Macrocystis pyrifera*, *Ecklonia kurome* and *Chorda filum* are more complex with the addition of different monosaccharides[20].

1.4.2 Structure

Till date there is no particular chemical structure of fucoidan has been established due to the variety of sources and many factors influencing the composition of fucoidan. Thus, the name fucoidan is directed towards general characteristics of the compound rather than a particular chemical structure (fucoidan composed of mainly fucose constitute the backbone of the structure and sulphate group attached to them creating branches). The term *sulphated fucan* in accordance to the IUPAC (International Union of Pure and Applied Chemistry) refers to a polysaccharide containing sulphated L-fucose with less than 10% (w/w) of other monosaccharides and the term fucoidan has been mainly used for seaweeds derived fucan[18].

Percival *et al.* investigated the structure and composition of fucoidan obtained from four different species: *Fucus vesiculosus, Fucus spiralis, Laminaria cloustoni* and *Himanthalea lorea*[19]. In this study; extraction with hot water, followed by alginate and protein removal with lead acetate, also with the addition of barium hydroxide; was used to obtain crude fucoidan. Hydrolysis of fucoidan using 0.5 N sulphuric acid for 3 hours was performed to obtain free fucose. It was found that, with the utilisation of polarimetric method, crude fucoidan from *H. lorea* contain 43.9% (w/w) fucose. The amount of fucose in *H. lorea* was the highest compared to *Fucus vesiculosus, F. spiralis,* and *Laminaria cloustoni;* with each of these samples contain 41.2% (w/w), 35.8% (w/w), and 36.5% (w/w) of fucose respectively. Other components found to be sulphate and some metals residues (calcium, magnesium, sodium and potassium). This crude fucoidan was then purified through several steps; dissolution in water, evaporation and re-precipitation in alcohol. After hydrolysis, further analysis using quantitative paper chromatography showed that not only the percentage of major component of fucose increased to 79.1% (w/w); some other sugars such as galactose 7.2% (w/w), xylose 1.3% (w/w) and uronic acid 2.8% (w/w) were also observed and were regarded as impurities.

Similarly, Schweiger *et al.* observed the existence of sugar or monosaccharide residues other than fucose in fucoidan. They extracted fucoidan from different species of seaweed. This study used *Macrocystis pyrifera*, a brown seaweed species, which is commonly utilised in the production of alginic acid or alginate. They found that, through filtration and crystallisation of L-fucose, the filtrate contained some portion of L-fucose and other components of sugars or acids. Galactose and xylose were observed through chromatographic technique, with also a small trace of mannose but no evidence of uronic acids[21]. Following methylation method and identification of methylated sugar after hydrolysis, they concluded that fucoidan is a fucan with mainly L-fucose residues linked by α-(1→2) glycosidic bonds and sulphate group attached in

the fourth position of L-fucose residues. There is also a possibility that every fifth fucose residue in the structure has a fucose branch attached on it[19]. This structure of fucoidan was accepted for almost 50 years before Patankar and colleagues offered a new structure and, they claimed, to be more probable structure.

Patankar *et al.* concluded that the availability of tri-*O*-methyl-L-fucose in fucoidan molecule has a very high degree of branching[22]. This was different to the branching site suggested previously by Percival *et al.* which was to be fucose 4-sulphate. It was proposed that the core chain of fucoidan to be α-(1→3) linked fucose and substituted with sulphate group at C-4 position of fucose. Results from sulphate analysis indicated that one sulphate group is present in the structure for every 3-4 mol of fucose (0.3 mol of sulphate/mol of fucose)[22]. These findings were similar to that observed by Ermakova and colleagues when attempted to determine the structure of fucoidan. It was suggested, based on the Nuclear Magnetic Resonance (NMR) analysis, that fucoidan from *Sargassum hornery* has a structure of α-L-fucopyranose linked in 1,3 position with sulphate attached at position 2, while *Costaria costata* showed a more complex structure with existing residues of acetyl groups, methyl groups and galactopyranose. Fucose in *Fucus vesiculosus* fucoidan is also attached to the main structure creating a branched site of two to three fucose residues in the molecule[11]. The fucoidan structures as suggested by Percival (A) and Patankar (B) are shown in Figure 2.

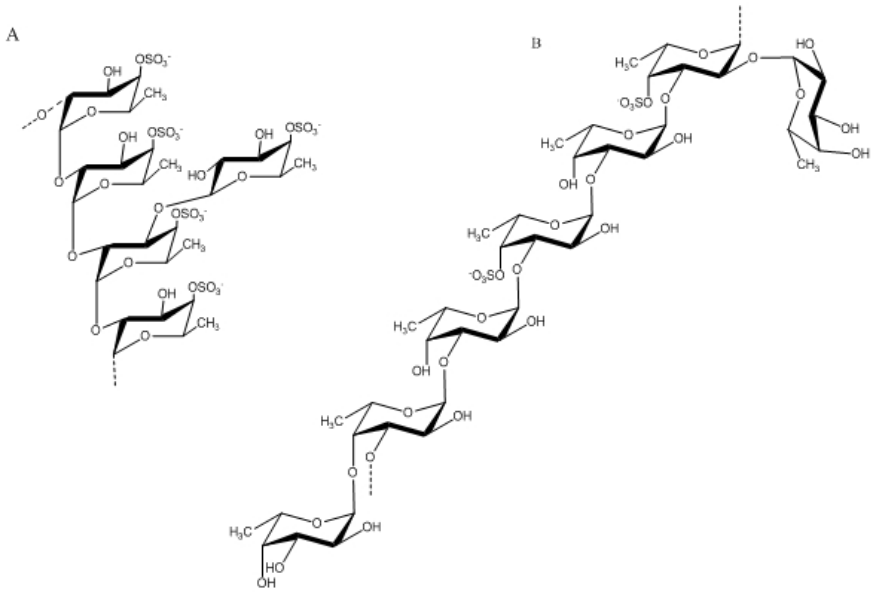

Figure 1.2 The proposed structure of fucoidan[23]

Different studies have also investigated the structure of fucoidan extracted from different seaweeds (summarised in table 1.1). It can be seen from the table that the structure of fucoidan and its compositions varies between one seaweed to another. This conclusion can be withdrawn, for instance, based on the results reported by Bilan *et al.* They extracted fucoidan from three different seaweeds: *Saccharina latissima*, *Fucus serratus L.*and *Chordaria flagelliformis*. The same fractionation method using anion-exchange chromatography and analysis using NMR spectroscopy were performed in all three studies. As shown in table 1.1, *Fucus serratus* derived fucoidan composed of L-fucose, sulphate and acetate with the molar ratio of (1:1:0.1), while that extracted from *Chordaria flagelliformis* was composed of L-fucose, sulphate and D-glucuronic acid with the molar ratio of (1:1:0.25).

The result of the studies listed in table 1.1 also suggested that there is a common structure of fucoidan observed in most of the investigated seaweeds disregard to the utilised degradation method. Fucoidan degraded with four different methods: fractionation using anion-exchange

chromatography, methylation, autohydrolysis and enzymatic hydrolysis; were identified to have the structure of 3-linked α-L-fucopyranose. This commonly exist L-fucose in fucoidan has been considered as a general characteristic in the identification of fucoidan as that also used by IUPAC as it is mentioned earlier in this chapter.

Table 1.1 Structural properties of fucoidan from different seaweeds and extraction methods

Source of seaweeds	**Structure of fucoidan and composition**	**Extraction methods**	**Reference**
Saccharina latissima	3-linked α-L-fucopyranose 6-linked β-D-galactopyranose 4-linked β-D-glucopyranosyluronic acid and 2-linked α-D-mannopyranose 3-linked β-D-glucopyranosyluronic acid	Fractionation using Anion-exchange chromatography with NMR spectroscopy analysis	Bilan *et al.*[24]
Fucus serratus L.	3- and 4-linked α-L-fucopyranose residues →3)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→ Fucoidan composed of L-fucose, sulphate and acetate (1:1:0.1)	Fractionation using Anion-exchange chromatography with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy analysis	Bilan *et al.*[25]
Chordaria flagelliformis	3-linked α-L-fucopyranose α-L-Fuc*f*-(1→2)-α-L-Fuc*f*-(1→ Fucoidan composed of L-fucose,, sulphate and D-glucuronic acid (1:1:0.25)	Fractionation using Anion-exchange chromatography with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy analysis	Bilan *et al.*[26]
Laminaria cichorioides	(1→3)-type α-L-fucopyranose	Autohydrolysis	Anastyuk *et al.*[27]
Analipus japonicus	1→3)-linked α-L-fucopyranose	Methylation, periodate oxidation coupled with NMR spectroscopy	Bilan *et al.*[28]
Fucus distichus	→3)-α-L-Fuc*p*-(2,4-di-SO_3^-)-(1→4)-α-L-Fuc*p*-(2SO_3^-)-(1→ Mostly disaccharides and no fucose nor other monosaccharides observed	Enzymatic hydrolysis using enzyme fom mollusc Littorina kurila	Bilan *et al.*[29]
Ascophyllum nodosum	Mostly α-(1→3)-linked fucosyl residues and a few α-(1→4) residues	Methylation, Smith degradation coupled with FTIR and NMR spectroscopy analysis	Marais *et al.*[30]

It has also been reported that there are two known types of fucoidan based on their glycosaminoglycan structure; F-fucoidan and U-fucoidan. F-fucoidan consists of more than 95% sulphated ester of L-fucose in seaweeds, while U-fucoidan is composed of approximately 20% glucuronic acid. The average molecular weight (MW) of fucoidan is also considered very high. Fucoidan from *Hizikia fusiformae,* for example, has an average molecular weight of 92.7 kDa, while molecular weight of *Fucus vesiculosus* derived fucoidan varies between 100 to 217 kDa and in *Erylus discophorus* found even higher of up to more than 2000 kDa[15,20,31].

1.4.3 Extraction and hydrolysing method

More than 60 years ago, hot water was used to extract crude fucoidan from several seaweeds species. This was followed with the addition of sulphuric acid to remove and decompose alkali-fucoidan complex. The alkali-fucoidan complex was filtered and acid was evaporated followed by precipitation of fucoidan using alcohol. Total of fucoidan yielded with this method was reported to be 79.1% of the dried sample as confirmed by quantitative paper chromatographic method[19]. Similar method employed isopropyl alcohol and methanol to extract fucoidan. A total of 110 g *purified fucoidan* was obtained from 330 g crude fucoidan. Fucose concentration in the purified fucoidan was 24.9% of the total sample and this was determined with the utilisation of oxidation reaction of fucose with Fehling solution[21]. Other methods used 0.03 M hydrochloric acid, papain, and a mixture of 4 M trifluoroacetic acid/methanol and water (4:2:1)[15,32,33]. It was reported that the amount of fucoidan yielded by each of these methods was 2.9%, 3.2% average and 1.75% of the dry weight seaweeds respectively.

Various techniques have been used to depolymerise or hydrolyse fucoidan into smaller molecular weight or structurally less complex oligosaccharides including chemical or enzymatic degradation. Attempts have been made to fractionate fucoidan from *Ascophyllum*

nodosum utilising ion-exchange displacement centrifugal partition chromatography. The separation was aided by running 0.025 M NaOH as mobile phase through a circular partition disk rotor column packed with ion-exchanger organic stationary phase of 10% solution of amberlite LA2 in methyl isobutyl ketone (MiBK). This method successfully fractionated fucoidan into smaller oligosaccharides with different molecular mass composition and sulphation pattern. The resulted low molecular weight (LMW) fucoidan was categorised into 3 groups based on the average molecular size; 9.5 kDa, 7.9 kDa and 7.1 kDa[34]. Another technique utilised for the fractionation of fucoidan was gel filtration chromatography. Using this technique fucoidan was separated into two major fragments, one having crude fucoidan containing 52.8% fucose and another consisted of LMW sulphated polysaccharide with 57.4% galactose[35]. LMW fucoidan was also obtained using chemical or enzymatic degradation methods. Chemical degradation of fucoidan was performed using either with 10 mL of 0.05 M sulphuric acid at 105°C for 5 hours or 1.5 mL concentrated 12 M sulphuric acid at 30°C for 1 hour which produced LMW fucoidan oligosaccharides with an average molecular weight of 43 kDa from the initial molecular weight of main sulphated polysaccharide of 680 kDa[36]. Other study used 2 M trifluoroacetic acid at 100°C for 7 hours to hydrolyse fucoidan and obtained oligosaccharides with average molecular weight ranged from 30 kDa to 80 kDa from the initial molecular weight of crude fucoidan of 710 kDa[37].

Depolymerisation of fucoidan was also carried out using *fucoidanase* enzymes which are commonly isolated from marine bacterium[38]. However, there are studies which utilised *fucoidanase* from sources other than marine bacterium for the degradation of fucoidan[39]. For instance, use of *fucoidanase* from sporophylls of *Undaria pinnatifida*, sea cucumber and *Fusarium sp.*[33,40,41]. Degradation of fucoidan using *fucoidanase* enzymes was thought to be more effective because they are known to cause site specific breakdown of linkage or bonding

in the fucoidan structure. Fucoidanase isolated from *Flavobacterium* sp., for example, was stable in a broad range of pH and able to degrade fucoidan resulting in low molecular mass of up to 1.3 kDa. The hydrolysates from enzymatic degradation of fucoidan were found to contain more than 98% of fucose[42]. *Fucoidanase* has also been isolated from a bacterium (which was then called as SW5) that inhabited mud in the water treatment facility of an alginate plant; which was added to 1 L of solution containing fucoidan from *Pelvetia canaliculata* and 20 mM Tris-HCl buffer [pH 7.5], 5 mM MgCl2, 5 mM CaCl2 and 50 mM NaCl. The degradation products were analysed by gas chromatography as well as high-performance anion-exchange chromatography (HPAEC) coupled with conductivity detection. The structural characterisation of fucoidan degraded fragments was carried out using NMR technique. It was found that the degradation products of fucoidan were composed of a tetrasaccharide sequence with a repetition structure of two units α-1→3-L-fucopyranose-2-sulphate and α-1→4-L-fucopyranose-2,3-disulphate, with the possibility of a residue (either sulphate, xylose, fucose or sulphated fucose) attached at C-4 position of fucose in the main structure. It also contained a hexasaccharide unit with the same homologous series[39]. Some other fractionation methods of fucoidan have also been listed above in sub-chapter 1.4.2.

1.4.4 Biological Activities

1.4.4.1 Anticoagulant

Seaweeds have been reported to be used for decades as a kind of traditional medicine and are thought to exert various biological activities such as anti-inflammation, antivirus, antibacterial properties and anticoagulant[4,43-48]. Fucoidan is known to be one of the polysaccharide components of seaweeds which is mainly composed of fucose. Therefore, in recent years, research focus is driven towards fucoidan to check whether or not fucoidan contains biological properties similar to seaweeds. One of the recognised biological properties of fucoidan is its

anticoagulant effect which is similar when compared to other high molecular weight, sulphated polysaccharides, such as unfractionated heparin (UFH)[43]. Fucoidan extracted from *Pelvetia canaliculata* showed anticoagulant effect, *in vitro,* at the concentration of 10 µg/mL. Similar to UFH, the anticoagulant effect of fucoidan from *Pelvetia canaliculata* was found to be because of the inhibition of factor IIa and Xa in the presence of heparin cofactor II. The efficacy of fucoidan was reported to be similar to UFH and dermatan sulphate in potentiation of heparin cofactor II. The anticoagulant effect of fucoidan from *Pelvetia canaliculata* was also compared to commercially available fucoidan having five times higher molecular weight and it was found that this commercially available fucoidan had no or little anticoagulant effect. It was postulated that high molecular weight of commercially available fucoidan was thought to influence its anticoagulant effect[43].

1.4.4.2 Anti-inflammatory

A study suggested that sulphated polysaccharide extracted from *Sargassum hemiphyllum* (at the concentration of 5 mg/mL) significantly inhibited the secretion of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and nitric oxide (NO), from lipopolysaccharide activated macrophage. The mechanism as to how this fucoidan inhibited the release of those pro-inflammatory cytokines was thought to be caused by the presence of polyphenols that suppressed the nuclear BF-κB receptor activity[44].

1.4.4.3 Anti-Human Immunodeficiency Virus (Anti-HIV)

The anti-HIV effect of *Fucus vesiculosus* derived fucoidan has also been studied. In a study, fucoidan oligosaccharides were obtained by running the crude fucoidan through a gel filtration chromatography and then the oligosaccharides were classified according to their molecular weights: high molecular weight, intermediate molecular weight and low molecular weight

oligosaccharide. Each high molecular weight and intermediate molecular weight oligosaccharide was further separated by anion exchange chromatography and then was tested for their anti-HIV effect. Some oligosaccharides inhibited both the activity of reverse transcriptase (RT) enzyme and syncytium formation while some of them only inhibited the RT enzyme but not the formation of syncytium. The anti-HIV effect was thought as the result of virus attachment to the CD4 receptor was blocked through the attachment of the highly negatively charged sulphate group to the basic V3 loop on the receptor. However, interestingly, this study also found that, the anti-HIV activity of fucoidan was not affected by the presence of absence of sulphate groups. These results suggested a possibility of a different mechanism by which these sulphated molecules displayed their anti-HIV effect[47].

Later, the anti-HIV effect was also studied and was found that the most probable active compounds are polysaccharides with high molecular weight (>2000kDa)[31]. Fucoidan was also able to inhibit adipogenesis mediated reduction of acid binding protein (aP2), acetyl CoA carboxylase (ACC) and peroxisome proliferation-activated receptor γ (PPARγ) and mRNA levels[49]. In another study, the extract containing fucoidan from *Fucus vesiculosus,* obtained by sequential extraction utilising diluted hydrochloric acid, has been reported to possess antioxidant effect when tested with ferric reducing antioxidant power (FRAP) and this activity was found to be stronger compared to that of sulphated galactan from red or green seaweed[36].

1.4.4.4 Anticancer

There are other biological activities of fucoidan that have also been studied and recognised, such as antitumor, immune-stimulatory, antibacterial, antiviral and anti-protozoan[50]. When introduced to the HCT 116 colorectal carcinoma cell line, *Ascophyllum nodosum* derived fucoidan (at the concentration of 1 mg/mL) alleviated the viability of HCT 116 colorectal

carcinoma cells. This effect was thought to be related to the ability of fucoidan in inducing apoptosis in cancer cells. Also, the activity was found to be dose dependent. For example, no significant apoptosis effect was observed when HCT 116 cells were treated with 300 μg/mL of fucoidan. However, this effect was evident in more than 57% of cells when treated with 1000 μg/mL of fucoidan[15]. A similar effect was also exhibited by fucoidan extracted from *Cladosiphon okamuranus* which exerted antiproliferative and markedly cytotoxic effects on MCF-7 cells, a breast cancer cell line, in a dose-dependent manner. Treatment of MCF-7 cells with fucoidan resulted in accumulation of sub-G1 population, chromatin condensation and internucleosomal fragmentation of deoxyribonucleic acid (DNA) which are the characteristic of apoptotic cell death[51].

1.4.4.5 Anti-diabetic

Another reported important biological activity of fucoidan is its effect in regulating blood sugar levels. This biological function has been studied but is still limited to *in vitro* or animal models. A study showed that, when administered to alloxan-induced diabetic mice, fucoidan significantly reduced the glucose levels which was partly related to an increase in insulin serum level. This effect was dose-related, as higher dose of fucoidan gave a larger reduction in glucose level[52]. In another study, LMW fraction of fucoidan, with an average molecular weight of 7 kDa, was found to be effective in attenuating diabetic retinopathy in streptozotocin-induced diabetic mice[53].

1.4.4.6 Structure-related bioactivities

This is, in some way, has been proven to be true. Similar to that of heparan sulphate and dermatan sulphate, glycosaminoglycans which contain sulphate group in their structure, biological activity (particularly on its anticoagulant property) of fucoidan is also influenced by

the amount of this sulphate group. Heparin sulphate and dermatan sulphate have shown that their antithrombotic activity is increased with increase in the number of sulphate groups. The resulphation method was aimed to produce a similar amount of sulphate group to that of heparin as a reference in observing the anticoagulant activity. Also, both antithrombotic and anticoagulant activities of these two glycosaminoglycans are based on the ability to catalyse the inhibition of thrombin and/or to inhibit the activation of prothrombin. The increased number of sulphate groups has enhanced the catalytic effects on the inhibition of thrombin by heparin cofactor II in plasma[54].

Similarly, *Ecklonia kurome* derived fucoidan with different molecular weight and the degree of sulphation was tested for its inhibitory effect on thrombin-fibrinogen reaction and amidolytic activity of thrombin. An increased in the inhibitory effect and amidolysis of the protein in the presence of heparin cofactor II was observed as a result of the increase of molecular weight. However, these activities were reduced with the decreased sulphate content. Further test showed no change in the binding ability of fucoidan to fibrinogen with the change of molecular weight and sulphate content. They suggested that heparin cofactor II-mediated antithrombin activity of the fucoidan was not depending on both molecular weight and sulphate content[55].

1.4.4.7 Low molecular weight (LMW) Fucoidan

Fucoidan is a branched polysaccharide with a very high molecular weight (HMW). It is composed of a highly negatively charged, polydispersed and unidentified oligosaccharides having fucose as the main component. Fucoidan has a high negative charge owing to the presence of sulphate groups. Fucoidan is also a mixture of small, medium and HMW oligosaccharides. Therefore, the biological activities of not only HMW but also LMW

oligosaccharides of fucoidan have been investigated. Some have also studied the biological activities between LMW fucoidan and unfractionated fucoidan having HMW.

High molecular weight oligosaccharides of fucoidan have been studied for their effects on attenuating N-nitrosodiethylamine-induced liver fibrosis. It has been found that these oligosaccharides exerted anti-fibrogenesis effect due to the down regulation of the transforming growth factor beta 1 and chemokine ligand 12 expression[56]. The influence of molecular weight on the anticoagulant effect was also observed and found that HMW fucoidan showed better anticoagulant effect *in vitro* while LMW fucoidan promoted a better effect *in vivo*[57]. A LMW fucoidan, extracted from *Laminaria japonica,* was found to be hepatoprotective when given to liver injury-induced CCl4 (Carbon tetrachloride) and D-galactosamine mice[58]. Another study compared the effectiveness of LMW fucoidan with calcium dobesilate against diabetic retinopathy in streptozotocin-induced mice as well as in a high glucose-promoted vascular endothelial growth factor (VEGF) cells. LMW fucoidan alleviated diabetic retinal neovascularisation likely through lowering hypoxia-inducible factor-1α (HIF-1α) and VEGF expressions[53].

Ermakova *et al.* investigated the structure-related activity of fucoidan using six different oligosaccharides of fucoidan extracted from three different species (*Eclonia cava, Sargassum hornery* and *Costaria costata).* The six extracted oligosaccharides were: ShF1 (sulphated fucan), ShF2 (non-sulphated fucan), ShF3 (sulphated rhamnofucan), EcF1 (sulphated rhamnogalactofucan), EcF2 (sulphated galactoglucofucan) and CcF1 (sulphated galactofucan). The effects of these oligosaccharides on the colony formation were investigated using human melanoma (SK-MEL-28) and colon cancer (DLD-1) cells. All the oligosaccharides but EcF2 showed inhibition to colony formation. EcF2 showed 8% inhibition compared to ShF2 and

ShF3 which significantly inhibited the formation of colonies. It was considered that the effects were probably influenced by sulphate groups, monosaccharide residue ratio and type of sugar residues bonding[11].

Table 1.2 summarises various biological properties possessed by either LMW or HMW fucoidan from different sources of seaweeds. It can be seen from the table that fucoidan extracted from different seaweeds can exert similar bioactivities. For instance, anticancer effect was shown by fucoidan extracted from *Turbinaria ornata* and *Ascophyllum nodosum*. The active fraction of *Turbinaria ornata* derived fucoidan was suggested to be the LMW fucoidan with (1→3)-linked fucose residues while that extracted from *Ascophyllum nodosum* was HMW fucoidan with the MW of up to 420 kDa[15,59]. This suggested that both LMW and HMW fucoidan can exerts similar biological activities even when they extracted from different source of seaweeds.

Table 1.2 Fucoidan isolated from different seaweeds, biological activity and the proposed active structure

Source of seaweeds	Biological activity	Types of the study	The proposed active structure of fucoidan	Reference
Pelvetia canaliculata	Anticoagulant	*In vitro*	LMW Fucoidan with 3- or 4-O-sulphate-L-fucose unit (MW of 20±5 kDa)	Colliec *et al.*[43]
Sargassum hemiphyllum	Anti-inflammatory	*In vitro*	Sulphated polysaccharides extract consisted of fucose, galactose, glucose, galactosamine, mannose, sorbitol and *myo*-inositol	Hwang *et al.*[44]
Fucus vesiculosus	Anti-HIV	*In vitro*	Both sulphated R-O-SO_3- and non-sulphated structure of LMW fucoidan (MW of 10-20 kDa)	Beress *et al.*[47]
Erylus discophorus	Anti-HIV	*In vitro*	HMW fucoidan extract with the polysaccharide content of up to 98%	Esteves *et al.*[31]
Eclonia cava *Sargassum hornery* *Costaria costata*	Anticancer	*In vitro*	Non-sulphated fucan and sulphated rhamnofucan from *S. hornery* extract (both consisted of fucose and rhamnose residue)	Ermakova *et al.*[11]
Saccharina cichorioides	Anti-tumour	*In vitro*	sulphated (1→3)-α-L-fucan	Ermakova *et al.*[60]
Turbinaria ornata	Anticancer	*In vitro*	LMW fucoidan with (1→3)-linked fucose residues	Ermakova *et al.*[59]
Ascophyllum nodosum	Anticancer	*In vitro*	Fucoidan extract (MW: 47 and 420 kDa) consisted of fucose, galactose, xylose and sulphate groups.	Foley *et al.*[15]
Cladosiphon okamuranus	Antivirus	*In vitro*	Sulphate groups and glucuronic acid of fucoidan	Hidari *et al.*[61]
Fucus vesiculosus	Hepato-protective	*In vivo*	HMW fucoidan consisted of fucose, galactose, xylose, uronic acid and sulphate groups	Hong *et al.*[45]
Cladosiphon okamuranus	Anti-fibrotic	*In vivo*	HMW fucoidan (MW: 41.4 kDa)	Nakazato *et al.*[56]
Sargassum patens	Antivirus	*In vitro*	HMW fucoidan (MW: 424 kDa) consisted of fucose, galactose, mannose, xylose, glucose and galactosamine	Zhu *et al.*[46]

Beress *et al.* investigated the anti-HIV effect of fucoidan extracted from *Fucus vesiculosus* and concluded that there was probably no particular structure influencing the bioactivities of fucoidan. The investigated effect was shown by both the tested sulphated and non-sulphated fucoidan fractions[47]. This was similar to that reported by Ermakova *et al.* in a study investigating the anticancer effect of fucoidan from *Eclonia cava, Sargassum hornery and Costaria costata.* They suggested that both non-sulphated fucan and sulphated rhamnofucan exert anticancer activity. However, among the tested fucoidan fractions from the three seaweeds, only non-sulphated fucan and sulphated rhamnofucan extracted from *Sargassum hornery* showed the anticancer effect[11]. The results provided by these studies suggested that, is some cases, the biological activities of fucoidan is independent to its molecular weight or the existence of sulphate groups. However, in other cases, fucoidan extracted from a particular seaweed exerts a certain bioactivity while it is not observed from fucoidan extracted from another type of seaweed(s).

1.4.5 Toxicology

Different studies have determined its toxicological factor or safety. The *Undaria pinnatifida* derived fucoidan at a dose of 1000 mg/kg body weight was suggested to be safe in rats following 28 days of administration[62]. Another clinical study showed that the oral administration of fucoidan (3 g daily) in healthy human volunteers did not show any significant adverse effects except the observed alteration in blood clotting time. However, this clotting time was still within the normal clinical range[63,64]. The effects of a more excessive ingestion of fucoidan (extracted from Mozuku seaweed) of up to 4.05 g daily, given to 20 subjects for 2 weeks, had also been investigated. There were no reported adverse effects on the subjects' health nor abnormalities observed in the abdominal, blood, urine and fecal states[65]. Therefore, orally administered fucoidan can be considered safe based on these studies.

1.5 Fucoidan Absorption: Uncertainty

Nowadays, fucoidan is available commercially because of its biological effects for the management of various disorders. It has been reported that fucoidan is relatively safe when given 3 g daily for 12 days[63]. However, fucoidan has a HMW and therefore its absorption into the systemic circulation following oral administration is questionable. The extent of fucoidan (either HMW or LMW) being absorbed into the systemic circulation, still needs to be investigated.

Physicochemical properties (such as molecular weight, p*K*a and lipophilicity) of a drug can influence its oral bioavailability. Along with physicochemical properties, there are several other parameters which can influence the bioavailability of a drug. These include solubility, gastric emptying, gastrointestinal pH and interaction of the drug either with food, mucus, GI enzymes or with other drugs. It is important to consider all these factors while investigating the systemic absorption of fucoidan. There are two things which have not been clearly determined regarding the absorption of fucoidan: the mechanism by which fucoidan is getting absorbed and whether or not it is degraded prior to the absorption, and the site or location in the gastrointestinal tract (GIT) for fucoidan to be absorbed. These create uncertainty in the absorption of fucoidan.

1.5.1 Mechanism of Absorption

Barthe *et al.* described four potential mechanisms that would help a drug to cross the epithelial cell layers in the GIT; passive diffusion through the membranes, carrier-mediated transport, endocytosis and paracellular transport. Most LMW lipophilic drugs cross the intestinal mucosa through passive diffusion and the absorption rate is proportional to the drug concentration and the surface area. Some drugs also utilise carrier-mediated transport which involves interaction

between the drug and a transporter. This mechanism is facilitated through either active transport, mediated by the use of metabolic energy, or passive transport which is driven by the concentration of the drug. It is suggested that macromolecules uptake (such as polysaccharides) in mammalian cells occurs with the utilisation of endocytosis mechanism and involves the endocytocytic vesicles with lysosomes containing high degree of enzymatic activity[66]. Chondroitin sulphate, an acidic mucopolysaccharide with considerably HMW, was suggested to utilise this endocytosis transport[67]. Paracellular transport, in a different way, occurs when a drug crosses the epithelial cell through a tight-junction and passes between the cells. Although, this tight-junctions cover only 0.1% of the intestine area, some small hydrophilic solutes, such as nutrients and ions, are able to cross through this site[68]. It is also known that some compounds, such as sodium caprate, chitosan and *N*-trimethyl-chitosan hydrochloride derivatives, have the ability to enhance the absorption of considered poorly absorbable drugs like peptides, peptides analogs or other hydrophilic macromolecules on this paracellular route[66,68].

The specific route via which oral fucoidan is absorbed, if absorbed at all, from the GIT is still unclear. General thought would consider fucoidan cannot be absorbed due to its very high molecular weight and hydrophilic properties. However, by comparison to other compounds which have similar properties, such as heparin, would give a better understanding of the probable mechanism for fucoidan to get absorbed.

Heparin and fucoidan are both highly sulphated polysaccharides. Heparin is recognised for its anticoagulant activity and is commonly given as a post-operative treatment to prevent deep vein thrombosis and pulmonary embolism[69]. Also, it is given to patients who undergo cardiac surgery using cardiopulmonary bypass, coronary angioplasty, vascular surgery and for patients with disseminated intravascular coagulation[70]. Heparin is categorised as glycosaminoglycan

and is derived from animal tissues[71]. Heparin is highly sulphated with *pentose-structured* monosaccharide as the main backbone and this is similar to that of fucoidan. The main difference, other than the type of sugar-backbone is that fucoidan has a more complex structure, with more branching site attached to its fucose backbone and higher molecular weight, compared to heparin. Heparin is a linear polysaccharide and composed of repeating monosaccharide units with commonly contains 1→4-linked residues of uronic acid[72].

The desire in developing oral heparin is increasing as it is considered to be more convenient compared to parenteral administration. On the one hand, because of its high molecular weight, hydrophilic and highly negatively charged structure; it is thought that heparin cannot be absorbed after oral administration. However, some studies have reported observable biological activity of heparin such as its antithrombotic or anticoagulant activity following oral administration. For example, orally given heparin in a dose of 7.5 mg/kg showed antithrombotic effect in rats compared to the control subjects given saline[73].

In another study, UFH which was given via intraduodenal route in rats also showed detectable anti-Xa activity in systemic blood and this activity peaked within 10 minutes of administration[74]. This suggested a possibility of heparin being absorbed after oral administration. Further investigation by Costantini *et al.* and Hiebert *et al.* utilising radiolabelled heparin demonstrated that heparin at some extents was absorbed and well distributed in the GIT tissues as well as reached blood circulation[73,74]. Costantini used ^{125}I-labeled heparin and observed this radiolabelled heparin was well distributed in the GIT vessel wall. The radiolabelled heparin also quickly reached blood circulation. The peak concentration of 10.6% (w/w) of the given dose was detected in peripheral blood and achieved within 5 minutes after administration. Moreover, the radioactivity in portal blood was found to be

higher than peripheral blood after 10 minutes of drug was given and, they concluded, this in line with the expectation that radiolabelled heparin was absorbed via the GIT mucosa. However, still in the same study, the peak plasma radioactivity was found to be 10 times lower when heparin was given via intragastric method and thought to be caused by the poor absorption on this site[74].

Similar result also obtained in different study by Hiebert and colleagues. They utilised ^{14}C-labelled heparin and was given to rats through oral route. Radioactivity was measured using ^{1}H-Nucleus Magnetic Resonance (^{1}H-NMR) spectroscopy to some collected specimens taken from parts of stomach, duodenum, jejunum, ileum, colon, lung, kidney, liver, and faeces. Radioactivity was observed in all of the specimens and suggested that radiolabelled heparin was readily distributed in the GIT vessels or gut tissues. Furthermore, although the concentration was 10- to 100-fold less compared to that in gut tissues, radioactivity was also observed in non-gut tissues. Total radiolabelled heparin detected in gut tissues reached an average of 31.1% compared to only 1.0% in non-gut tissues. The peak plasma concentration of radiolabelled heparin was achieved within 3 minutes after administration at 2.6% of the dose given[73].

The above studies conducted by Costantini *et al.* and Hiebert *et al.* presented the fact that heparin, despite its considered high molecular weight and negatively charged structure, was absorbed and distributed in the GIT tissues as well as reach blood circulation. Although the given drug was considered as derived heparin, both authors claimed either chemical and pharmacological properties of the radiolabelled heparin retained the original properties of heparin[73,74]. Nonetheless, it is also apparent from both studies that, although heparin is absorbed after oral administration, the total plasma concentration is very low. Hence, different

studies provided a formula to increase the absorption of oral heparin; one of which by the addition of other components carrying a function as absorption enhancer such as deoxycholic acid (DOCA), papain, Sodium *N*-[8-(2-Hydroxybrnzoyl)Amino] Caprylate (SNAC) and polyaminomethacrylate coacervates[69,75-77].

Fucoidan in many ways similar to heparin. Both fucoidan and heparin are polysaccharides constituted mainly with a particular monosaccharide, fucose for fucoidan and uronic acid for heparin, bound to one another with glycosydic bonding. They are highly negatively charged compounds as a result from the existence of sulphate structure attached to the main backbone creating branches. HMW is also one of the characterisics of fucoidan and heparin. Hence, these three similar characteristics between fucoidan and heparin could be considered as the starting point in predicting the fate of fucoidan after oral administration.

The first thought arises questioning whether fucoidan is broken down in the GIT to produce smaller oligosaccharides or it retains its whole initial big molecular structure. The ability of fucoidan to retain its initial molecular structure can possibly rely on the strength of the glycosidic bond connecting its smaller mono- or oligosaccharides fractions. The strength of this glycosidic bond could be referred to a study by Kenneth *et al*. on the stability of heparin in acidic and basic condition using 0.1N hydrochloric acid and 0.1N sodium hydroxide[78].

Kenneth *et al.* utilised gradient polyacrylamide gel electrophoresis (PAGE) and staining method and observed a reduction in the molecular weight of heparin under these strong acid and basic test conditions. An increase in reducing-end product from heparin was confirmed by PAGE method to be resulted from the glycosidic linkage breakdown of heparin molecule under acidic environment. Still with the same method, under strong acid stress test, heparin lost some

degree of its sulfation level. Under acidic condition of 0.1N hydrochloric acid in 60 °C for 1000 hours of treatment, heparin lost around 12% of its sulphate group from its molecule[78].

The *in vitro* test result reported by Kenneth *et al.* aligns with the finding that orally administered heparin was broken down into its smaller mono- or oligosaccharide. It was suggested that two pharmacological effects of heparin as being measured by thrombin activity and anti-factor Xa activity depend on its molecular size. While anti-factor Xa activity relates to the existence of fragments of heparin as small as pentasaccharide, thrombin activity is associated with oligosaccharides of at least tetradodecasaccharides. Since only anti-factor Xa activity was observed and no apparent thrombin activity following oral administration of heparin, this ended up to the conclusion that heparin was degraded to smaller fragments[74,79].

Still in the same study, Grabovac *et al.* tested two types of radiolabelled heparin, [^{3}H]heparin and [^{35}S]heparin, which was orally administered to rats. In regards to the type of isotope being used, [^{3}H]heparin is heparin which only is labelled at the reducing end while [^{35}S]heparin is evenly labelled throughout the molecule. The result showed that 10% recovery of radioactivity of [^{3}H]heparin in the urine was lower compared to 17% for [^{35}S]heparin. This strengthened the conclusion to the occurrence of degradation of heparin in the GIT; as if particular fragments of heparin were released into the systemic blood circulation, only the reducing end of [^{3}H]heparin would be radiolabelled but the fragments generated by [^{35}S]heparin would always be radiolabelled[79].

Taking into consideration the result of the studies conducted by Kenneth *at al.* and Grabovac *et al.*, fucoidan can also undergoes glycosidic bond cleavage in acidic gastric environment. This will cause fucoidan to subsequently lose some of its negative charge due to desulfation

and to produce smaller oligosaccharides and increase the possibility of absorption to occur. However, an important thing to note that, unlike heparin, fucoidan is not a linear in structure. It is more complex with higher degree of branches and higher molecular size. These factors can possibly hamper or at least minimise the effect of the degradation and desulfation caused by gastric acid.

Another important factor with the ability to degrade fucoidan into its smaller oligosaccharide is α-L-fucosidase enzyme which exists in the body. This enzyme could be found everywhere in human tissues and body fluids and has a role in catalysing the release of L-fucose from simple or complex polysaccharides[80]. However, similar to the influence of gastric acidic environment to fucoidan; the activity of this enzyme in degrading orally-given fucoidan to produce smaller oligosaccharides is yet to be determined.

The only available information about the absorption of oral fucoidan is those reported by Tokita *et al.* and Irhimeh *et al.* in their study[63,81]. Tokita *et al.* claimed the orally given fucoidan detected in plasma sample was remained unchanged. They utilised size exclusion chromatography with refractometry index detector (SEC-HPLC-RI) to compare the molecular weight of the given fucoidan to that detected in plasma and urine. They found out, the molecular weight of fucoidan in plasma was relatively the same to the administered fucoidan while in urine it was smaller. They then concluded that fucoidan was possibly degraded in secretory system but not in the intestine[81].

Irhimeh *et al.* in a similar study also confirmed the likelihood that fucoidan was not degraded following oral administration before it is absorbed into systemic blood circulation[63]. Tokita and Irhimeh also used Enzyme-linked Immunosorbent Assay (ELISA) method to detect fucoidan

in the plasma. Both studies confirmed that fucoidan was absorbed following oral administration. However, very low concentration of fucoidan detected in plasma; 0.6% of the given dose observed by Irhimeh while Tokita observed the plasma concentration of fucoidan to be 100 to 1000 times lower. They argued that this discrepancy occurred due to the different dose of oral fucoidan given in both studies, 3 times of 1 g fucoidan a day for 12 days and 1 time for 1 day respectively. Fucoidan concentration in plasma reached its peak after 6 to 9 hours after administration while in urine after 3 to 6 hours of administration[81].

Both studies by Tokita and Irhimeh showed a promising results indicating fucoidan, as it is heparin, is absorbed after oral administration. However, the fact that they suggested fucoidan was not broken down in the GIT, which is on the opposite to that of heparin, sparks questions. While this could be true and align with the thought that the higher molecular weight and more complex structure of fucoidan, compared to heparin, would probably limit the effect of enzyme and gastric acid environment to degrade its structure; some factors in these studies worth to get attention.

The first thing to note with regards to the absorption of fucoidan based on the result reported by Irhimeh *et al.* and Tokita *et al. is* the conclusion suggesting that orally given fucoidan retained its structure following absorption in the GIT. This was determined by comparing the molecular weight of the given fucoidan to that detected in plasma and urine sample. SEC-HPLC was used to determine the molecular weight of fucoidan. The molecular weight of fucoidan in the plasma was 55-96 kDa and in urine was much smaller with 1.8-3.1 kDa, while the orally administered fucoidan was 66 kDa in molecular weight[81]. It is understandable that the method was not able to determine the exact molecular weight of fucoidan; hence it is showed in average or range value of molecular weight. This could be caused by the fact that

fucoidan is a polydispersed molecule, without an exact entities. However, it also showed the lack of ability of this method to determine whether the lower detected molecular weight of fucoidan was caused by the polydispersed property of this molecule or due to the loss of some fractions from the structure.

Secondly, the fractionated fucoidan as a result from the SEC-HPLC method was then determined using ELISA for fucoidan structure detection. Given the understanding that the antibody in ELISA will only recognise particular part of the molecule of fucoidan, the loss of other fractions from the molecule will not be detected. For example, the utilised fucoidan antibody by Tokita was expected to react with the polymeric structure of fucose in fucoidan[81]. Hence, this antibody will only recognise and react with fucose fraction in fucoidan. It will not recognise or consider, for example, the existence of sulphate group attached to the structure to give the detection. As a result, even with the loss of this sulphate fraction from the molecule, the method would still give the detection of fucoidan. This could be the down side given by this method since desulfation, the loss of sulphate group as it occurs to heparin, would also possibly occur to fucoidan in the GIT. The method can also interact with endogenous fucose. Therefore, it also can probably interact with endogenous heparin and other polysaccharides.

Third, both studies by Irhimeh and Tokita focused on determination of the absorption of fucoidan following oral administration. There were no further attempts to include determination on the particular expected pharmacological effects of fucoidan, such as anticoagulant effect, which could also be used as confirmation to the absorption to occur. Some studies indicating the relation between the structural characteristics of fucoidan such as the molecular size and sulphate group with its biological properties. For example, molecular size of fucoidan was found to influence its anti-HIV activity of fucoidan[31]. In another study, the

branched structure of fucoidan (characterised with the existence of sulphate group and glycosidic bonds) showed to play an important role in achieving effective interaction with the complement protein which is a common mechanism for a drug to produce its pharmacological effects[82]. Measuring the pharmacological outcome as a part of determining the absorption of fucoidan could provide supporting evidence in taking the conclusion whether or not fucoidan is being absorbed, due to the correlation between the structure and its pharmacological activities as mentioned above.

The conclusion made by Tokita *et al.* and Irhimeh *et al.*, suggesting fucoidan is not degraded into smaller oligosaccharide after oral administration, could be true based on their study. However, a further study would help to provide more evidence to support this conclusion. This is because the conclusion was on the opposite to that of heparin which in some studies has been showed to be fractionated into smaller oligosaccharide in the GIT. The matter of fact, fucoidan has structure similarities with heparin.

Another important factor on the absorption of oral fucoidan is the mechanism taken to cross the intestine barrier and reach the systemic blood circulation. There were no clear explanations in the available studies on fucoidan about this. Nonetheless, it was suggested that fucoidan possibly utilised the endocytosis mechanism similar to that was suggested for chondroitin sulphate[63,67]. Further studies, however, are still needed to support this conclusion.

1.5.2 Site of absorption

It was reported that orally administered fucoidan need longer time to reach blood circulation; 3 hours for fucoidan compared to 5 minutes for heparin[74,81]. The peak plasma concentration was also very low; 0.6% (w/w) of the given dose when it was given 3 times of 1 g fucoidan a

day for 12 days[63]. Plasma concentration of the orally given heparin, though also very low of up to 10.6% (w/w) of the given dose, was higher compared to that of fucoidan[74]. Nevertheless, these studies have showed fucoidan is absorbed in the GIT. Unfortunately, there was no further information provided as to which mechanism play a role in the absorption of fucoidan and on which part in the GIT fucoidan is getting absorbed. Similar approach could reasonably be taken, to the available studies on heparin, to possibly predict the mechanism of fucoidan absorption and the site of absorption in the GIT.

Orally administered heparin has been proven to be absorbed in the GIT. Although the amount of the absorbed heparin detected in plasma was low, it was very well distributed in the cell walls or epithelium of the intestine prior to the absorption[73,74]. The absorption of heparin occurred either through gastric cell walls and the intestine[73]. The influence of molecular weight and pH to the heparin movement through gastric mucosa has also been studied. It was found that the molecular weight of heparin and pH in the gastric play important role to the amount of heparin being absorbed[71].

Two different types of heparin, unfractionated heparin (UFH) which is considered to have high molecular weight and LMW heparin (LMWH) with lower molecular weight, was tested for their absorption in the gastric of rats subject. As the pH was changed between pH 7.4 and pH 4.0, both UFH and LMWH showed different behaviour regarding its absorption through the gastric mucosa. The unfractionated heparin was suggested to easily cross the barrier in more acidic environment while LMWH was on the opposite. Bigger molecular size of the UFH was thought to prevent ionisation at acidic pH compared to LMWH and made it easier to cross the gastric mucosa. On the other hand, acidic environment from the lower pH gave less influence to the LMWH due to its lower molecular size. This also was applicable in the opposite way

when the test was done in basic environment at pH 7.4. Another factor influencing the degree and time of absorption of heparin, as suggested by Moazed *et al*. the author of the study, is the binding interaction between heparin with protein and cells in the mucosa. Compared to LMWH, UFH can interact with varieties of proteins. This will then prolong the time for UFH to remain stay in the luminal mucosal membrane before it is released to serosal side and probably reach the systemic circulation[71].

Taking the result of the study reported by Moazed *et al.* into consideration, fucoidan can also probably be absorbed through both gastric mucosa and the intestine. If similar result on heparin is also applied to fucoidan, there is a chance for fucoidan to get absorbed via gastric mucosa due to its very high molecular weight which was suggested to influence absorption in that site. Also, the possibility of the high interaction between fucoidan with protein and cells in mucosal membrane can give the answer to the lower plasma concentration and the longer time needed by fucoidan to reach blood circulation. However, this conclusion is made by the assumption that structural similarities between fucoidan and heparin will probably also give similar figure of its absorption in the GIT. Further study on fucoidan itself is needed to get the exact result.

1.6 Aims and Objectives

Interest in using fucoidan as a medication agent to deal with certain types of illnesses is increasing since it has been proven that fucoidan exerts particular biological activities. Fucoidan is used orally. However, no substantial evidence suggest that it is absorbed after oral administration. Some studies, using ELISA method, have suggested that it is absorbed. Additionally, other data showing fucose level in healthy and malignancy condition is also available. However, the utilised method had some problems such as cross-reaction as has been

mentioned above. Therefore, the objectives of this study are to develop a method which can determine the absorption of fucoidan and include

1. development and validation of a method capable in detecting and quantifying fucose level in the plasma
2. development of an extraction method to extract fucoidan from patients' plasma sample and a digestion method to breakdown fucoidan into fucose
3. analysis and quantification of fucose level in the patients' plasma samples using the developed method

It is expected that the result of the research will give a valuable data about the absorption profile of fucoidan in the GIT following single oral dose administration. This data is important to determine whether or not fucoidan or its fraction is absorbed. An evidence of increasing fucose level in the plasma will be considered that fucoidan probably undergoes digestion mechanism in the GIT that breaks fucoidan into its smaller fractions and then absorbed and passed through to the blood circulation. Also, the developed method will offer accuracy, sensitivity and validity in analysing fucoidan in plasma samples.

Chapter 2 - Materials and Methods

2.1 Materials and Instrumentation

2.1.1 Reagents

Unless otherwise specified, this study used HPLC grade Acetonitrile (ACN) which was purchased from ThermoFischer Scientific (Waltham, MA, USA). Water was purified with Milli-Q water purifying system with equipped ultraviolet lamp DirectQ UV3 and was filtered through 0.22 MilliPack filter from Millipore (Bedford, Massachusetts, USA). L-(-)-fucose, D-(-)-fructose, D-(+)-glucose, D-galactose and D-xylose were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia). Trifluoroacetic acid (TFA), Ammonium sulphate, ethanol (EtOH) and acetic acid were of analytical grade and purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia). Protamine sulphate was obtained from Sandoz (Boucherville, Quebec, Canada). Ammonium acetate was purchased from Fluka (Castle Hill, New South Wales, Australia. *Undaria pinnatifida* fucoidan (batch number UPF2012550) was obtained from Marinova Pty Ltd (Hobart, Tasmania, Australia) and was claimed to contain 87.2% fucoidan based on its Certificate of Analysis (COA). Blank plasma was obtained from Royal Hobart Hospital and Red Cross (Hobart, Tasmania, Australia).

2.1.2 HPLC instrumentation

Unless stated otherwise, a series of Dionex Ultimate 3000 RS HPLC instrument modules from Dionex (Sunnyvale, CA, USA) was utilised. A Dionex Ultimate 3000 RS pump with an internal degasser was used to pump and automatically mix mobile phase from separate chambers. Samples were auto-injected and the temperature was constantly maintained with a Dionex Ultimate 3000 RS Autosampler. A Dionex Ultimate 3000 RS Column Compartment was employed to keep the column at a stable temperature. The analyte of interest (fucose) was

detected using a Dionex Corona Ultra RS Charged Aerosol Detector (CAD). A Dionex Diode Array Detector and UV-Vis detector were also used during different methods trial. Chromeleon 7 Chromatography Data System was also used to control the operations of the instrument and to run data acquisition. Instrument diagram is shown in Figure 2.1.

2.1.3 Other instruments

A vacuum evaporator, miVac (Genevac Ltd, Suffolk, UK), was utilised to evaporate the plasma samples. Evaporation mode was set to H_2O mode and temperature was maintained at 30 ℃. The pH of the mobile phase was read using pH-meter EZDO PL-600 series which has been calibrated at the pH of 4.01, 6.83 and 9.01 with the appropriate buffer solutions each time before its use. A heating block, brand Multi-block, from Lab-line (Thermo Fisher, Waltham, MA, USA) was utilised during the digestion of samples. When necessary, samples were also mixed using Lab-line Super-mixer vortex equipment and underwent centrifugation using a Sky-line ELMI-6M centrifuge (ELMI, Riga, Latvia).

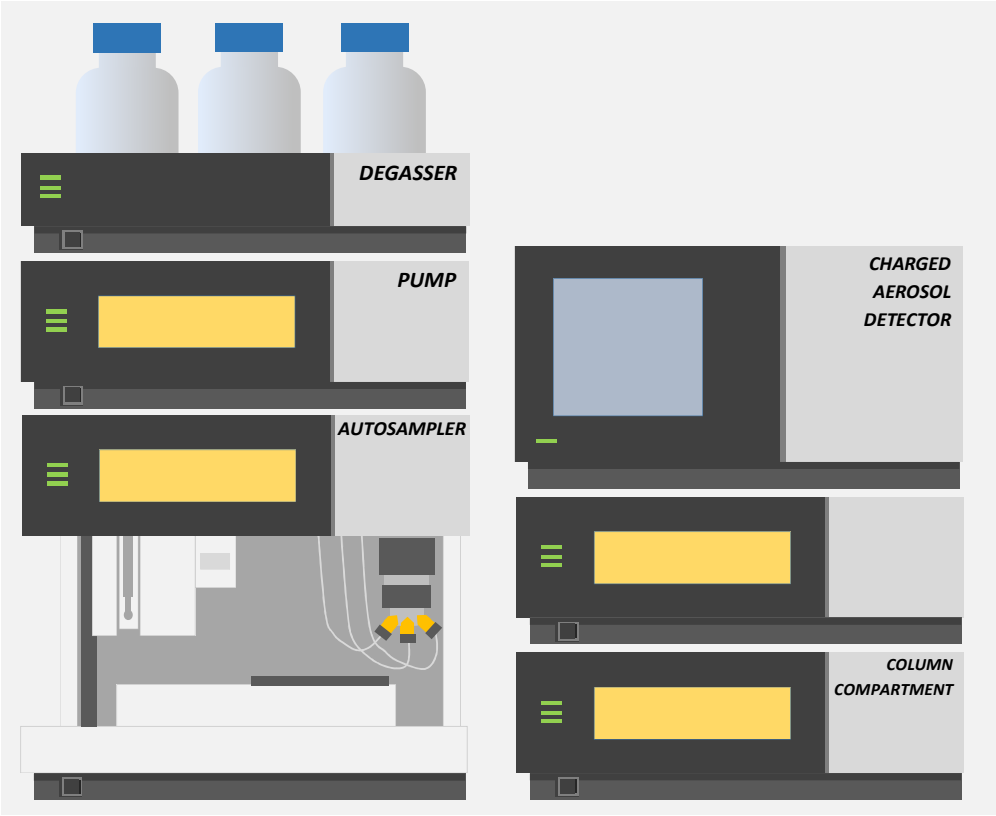

Figure 2.1 High Performance Liquid Chromatography Instrumentation

2.2 Experimental procedures

2.2.1 Sample preparation

2.2.1.1Preparation of Standard solutions

All the standard solutions were prepared by dissolving monosaccharides (fucose, glucose, fructose, xylose or galactose) with MilliQ water in a 10 mL separate volumetric flask. Each monosaccharide (10 mg) was weighted and transferred into a volumetric flask. Into this flask, 5 mL of MilliQ water was added. The flask was then kept in an operating ultra-sonic bath for 10 minutes or until the monosaccharide was completely dissolved. Subsequently, more water was added into the flask to make the total volume to be exactly 10 mL. The final concentration of each standard solution was 1 mg/mL and considered as the stock solution. Standard solution containing, 5, 10, 25, 50 or 100 µg/mL of each monosaccharide was made from this stock

solution through serial dilution using MilliQ water. This solution was prepared in triplicate and analysed once for each concentration.

2.2.1.2 Blank plasma sample

Blank plasma (500 µL) was taken and transferred into a 15 mL Corning (Corning, New York, USA) polypropylene centrifuge tube. This plasma was then precipitated using either ammonium sulphate, ethanol, 80% acetonitrile or acetonitrile as described below (2.2.3). The supernatant was carefully taken and discarded while the precipitate was kept intact. Then the digestion of precipitates was carried out using one of the three digestion solutions: concentrated sulphuric acid, 2M TFA or 20% TFA. Into the tube containing precipitate, the digestion solution was added and digestion was performed as described below (2.2.4). Subsequently, the mixture was evaporated to dryness with a vacuum evaporator. Milli Q water (1 mL) was then added into the dried sample, vortex-mixed for 1 minute and centrifuged at 3500 rotation per minute (rpm) for 20 minutes. The supernatant was taken and subjected to HPLC analysis. Each sample was prepared in duplicate and analysed in triplicate.

2.2.1.3Fucose-spiked plasma

The fucose standard solution was made by diluting 1 mL of fucose stock solution (1 mg/mL) with 9 ml of MilliQ water to obtain final volume of 10 mL. Plasma (500 µL) was pipetted and transferred into a 15 mL centrifuge tube. This plasma was then subjected to precipitation using the precipitation chemicals listed above. The supernatant was carefully taken and discarded while the precipitate was kept intact. Into the tube containing precipitate, 10, 20 50, 100, 200, 400 or 800 µL of fucose solution (100 µg/mL) was added to obtain final mixture containing 1, 2, 5, 10, 20 40 or 80 µg of fucose. The mixture was then evaporated to dryness. The dried sample was then subjected to digestion using the digestion chemicals described above.

Subsequently, the mixture was again left to evaporate to dryness in a vacuum evaporator. MilliQ water (1 mL) was then added to the dried sample, vortex-mixed for 1 minute and centrifuged at 3500 rpm for 20 minutes. The supernatant was taken to be analysed with the HPLC. Each sample was prepared in duplicate and analysed in triplicate.

2.2.1.4Fucoidan stock solution

Undaria pinnatifida extract powder (10 mg) was weighted and transferred into a 10 mL volumetric flask. Into this flask, 5 mL of MilliQ water was added and then sonicated using an operating ultra-sonic bath for 15 minutes or until all the powder was completely dissolved. MilliQ water was added into the tube to adjust the volume to 10 mL. This solution was considered as the fucoidan stock solution.

2.2.1.5Quality control sample

Blank plasma (500 µL) was taken and transferred into a 15 mL centrifuge tube. This plasma sample was then precipitated as described above**.** The supernatant was then completely taken and discarded while the precipitate was kept intact. Into the tube containing precipitate, 200 µL of fucoidan stock solution was added. This mixture was then underwent evaporation to dryness. This dried sample was then subjected to digestion as described above. Subsequently, the mixture was again left to evaporate to dryness in a vacuum evaporator. The dried sample was then dissolved with 1 mL of MilliQ water, vortex-mixed for 1 minute and centrifuged at 3500 rpm for 20 minutes. The supernatant was taken and considered as the final solution to be analysed with the HPLC. Each sample was prepared in duplicate and analysed in triplicate.

2.2.2 Solubility tests

The solubility of fucose and fucoidan in different solvents; ethanol, 80% acetonitrile, acetonitrile, acetone and ammonium sulphate; was investigated. Fucose and fucoidan powder and solution was mixed with 1, 2, 3, 4 or 5 mL of each of the solvents. The solubility was determined by visual observation of whether or not fucose or fucoidan precipitated after addition of the solvent.

2.2.3 Precipitation methods

2.2.3.1 Precipitation using ammonium sulphate

Precipitation of plasma protein using ammonium sulphate was tried in two consecutive steps. The first step was done by adding ammonium sulphate saturated solution into the plasma and the second by adding ammonium sulphate crystal.

The ammonium sulphate saturated solution was prepared, in the laboratory, by dissolving ammonium sulphate with Milli-Q water. A glass beaker was filled with 200 mL of water and kept on an operating magnetic stirrer. Ammonium sulphate crystal was slowly and gradually added into the beaker until when the further added ammonium sulphate was not dissolved.

Ammonium sulphate saturated solution (500 µL) was added into a centrifuge tube containing an equal volume of blank plasma. The mixture was then vortex-mixed for 1 minute and then centrifuged at 3500 rpm for 15 minutes. The supernatant (500 µL) was taken and transferred into a 15 mL centrifuge tube. Into this tube, 100 mg of ammonium sulphate powder was added, vortex-mixed for 1 minute and again centrifuged at 3500 rpm for 15 minute. The supernatant was carefully taken and considered as the final solution to be analysed with HPLC.

2.2.3.2 Precipitation using ethanol

Plasma protein precipitation using ethanol was tried using two different methods. The first method was referred to the work which was conducted by Subhash *et al*. using 95% ethanol[83] and the second has been previously done by Jianguo *et al*. utilising cold absolute ethanol[84].

After using different composition of blank plasma and 95% ethanol (1:1, 1:2, 1:3, 1:4 and 1:5 part volume of plasma to ethanol, respectively) it was found that the best precipitation was achieved using 1 volume of plasma and 4 volumes of ethanol. Therefore, blank plasma (1 mL) was taken and transferred into a 15 mL centrifuge tube. Into this tube, 4 mL of 95% ethanol was added, vortex-mixed for 1 minute and then centrifuged at 3500 rpm for 20 minutes. The supernatant was then taken and subjected to HPLC analysis. Each sample was prepared in duplicate and analysed in triplicate.

On the other hand, precipitation using cold ethanol was done by adding 11 mL of cold absolute ethanol (previously stored in 4 ℃ fridge) into 1 mL of blank plasma. The mixture was then vortex-mixed and stored at -20 ℃ overnight. Subsequently, the mixture was centrifuged at 3500 rpm for 20 minutes. The supernatant was then taken and analysed with HPLC. Each sample was prepared in duplicate and analysed in triplicate.

2.2.3.3 Precipitation using 80% acetonitrile

In this method the plasma proteins from one part of blank plasma was precipitated using four parts of 80% acetonitrile. Plasma (1 mL) was taken and transferred into a 15 mL centrifuge tube. Into this tube, 4 mL of 80% acetonitrile was added, vortex-mixed for 1 minute and then centrifuged at 3500 rpm for 1 minute. The supernatant was then taken and subject to HPLC for the analysis. Each sample was prepared in duplicate and analysed in triplicate.

2.2.3.4 Precipitation using acetonitrile

In this method, the plasma protein was precipitated using acetonitrile. Blank plasma (500 µL) was transferred into a 15 mL centrifuge tube. Into this tube, 2 mL of acetonitrile was added, vortex-mixed for 1 minute and then centrifuged for 20 minutes at the speed of 3500 rpm. The precipitate was kept and the supernatant was discarded.

2.2.4 Digestion method for plasma protein precipitate

The following methods were tested in the digestion of plasma protein precipitate.

2.2.4.1 Digestion using Sulphuric acid

Protein precipitate obtained from precipitation step was digested with concentrated sulphuric acid as has been described previously[85]. Sulphuric acid (1 mL) was added into a glass vial containing protein precipitate. The vial cap was tightly closed and sealed with paraffin film. Using a heating block with the temperature of 100 °C, this vial was heated for 2 hours. The mixture was then evaporated to dryness. Subsequently, 1 mL of MilliQ water was added into the dried sample and vortex-mixed for 1 minute. The mixture was then filtered using 0.22 µm syringe filter (Phenomenex, Lane Cove, NSW, Australia) and the filtrate was collected and subjected to HPLC analysis.

2.2.4.2 Digestion using 0.2M trifluoroacetic acid

TFA (0.2M, 3 mL) was added into a centrifuge tube containing protein precipitate obtained from precipitation stage using the method describe previously[86]. The tube's cap was tightly closed and sealed with seven to eight layers of paraffin film. Using a heating block, the tube was heated at 100 °C for 6 hours. The mixture was vortexed every 1 hour. After heating for 6

hours, the mixture was kept on open bench for at least 30 minutes. Once it reached the room temperature, the mixture was evaporated to dryness using a vacuum evaporator. Each sample was prepared in duplicate and analysed in triplicate.

2.2.4.3 Digestion using 20% trifluoroacetic acid

Digestion of glycoproteins was also performed using 20%(v/v) TFA as described previously[87]. TFA (3 mL of 20%) was added into the tube containing protein precipitates obtained from the precipitation step using acetonitrile as described in **2.2.3.4**. The tube's cap was then tightly closed and sealed with seven to eight layers of paraffin film. Subsequently, the tube containing mixture of glycoprotein and 20% TFA was put on a heating block and heated at 100 °C for 6 hours with vortexed mixing every 1 hour. Then, it was kept on open bench until it reached room temperature. A vacuum evaporator was then used to evaporate the solution to dryness.

2.2.5 Digestion method for fucoidan

The following methods were tested in the digestion of fucoidan.

2.2.5.1 Digestion using 0.2M trifluoroacetic acid

Digestion of fucoidan was performed using 0.2M TFA as described previously[86]. Fucoidan (15 mg) was weighted, transferred into a 20 mL volumetric flask. MilliQ water (10 mL) was added, then underwent sonication for 15 minutes or until fucoidan was completely dissolved. MilliQ water was then added to volume and thoroughly mixed. The solution (1 mL) was taken and transferred into a 15 mL centrifuge tube and was evaporated to dryness. TFA (0.2 M, 3 mL) was added into the dry sample. The tube's cap was tightly closed and sealed with seven to eight layers of paraffin film. Using a heating block, the tube was heated at 100 C for 6 hours. The mixture was vortexed every 1 hour. After heating for 6 hours, the mixture was kept on

open bench for at least 30 minutes. Once it reached the room temperature, the mixture was evaporated to dryness using a vacuum evaporator. MilliQ water (4 mL) was added into the tube containing the dry sample, vortex mixed for 2 minutes. The mixture was then underwent centrifugation for 20 minutes under 3000 rpm. The supernatant was carefully taken and subjected to HPLC analysis. Each sample was prepared in duplicate and analysed in duplicate.

2.2.5.2 Digestion using 20% trifluoroacetic acid

Digestion of fucoidan was also performed using 20%(v/v) TFA as described previously[87]. Fucoidan (15 mg) was weighted, transferred into a 20 mL volumetric flask. MilliQ water (10 mL) was added, then underwent sonication for 15 minutes or until fucoidan was completely dissolved. MilliQ water was then added to volume and thoroughly mixed. The solution (1 mL) was taken and transferred into a 15 mL centrifuge tube and was evaporated to dryness. TFA (20%, 3 mL) was added into the dry sample. The tube's cap was tightly closed and sealed with seven to eight layers of paraffin film. Using a heating block, the tube was heated at 100 C for 6 hours. The mixture was vortexed every 1 hour. After heating for 6 hours, the mixture was kept on open bench for at least 30 minutes. Once it reached the room temperature, the mixture was evaporated to dryness using a vacuum evaporator. MilliQ water (4 mL) was added into the tube containing the dry sample, vortex mixed for 2 minutes. The mixture was then underwent centrifugation for 20 minutes under 3000 rpm. The supernatant was carefully taken and subjected to HPLC analysis. Each sample was prepared in duplicate and analysed in duplicate.

2.2.6 HPLC analysis

2.2.6.1 Reversed phase chromatography with Tributylamine

Reversed phase chromatography using a C_{18} column, Grace Apollo C18 5 µm (250 mm, 4.6 mm ID) was used to resolve fucose. A security guard column (FC-2000 cartridge, Phenomenex,

NSW, Australia) was attached to the inlet of the column. Linear gradient of mobile phase consisted of 7.5 or 15 mM Tributylamine (TBA) in acetonitrile and 7.5 or 15 mM TBA in water with constant flow rate of 1.0 mL/minute was used. Mobile phase was filtered using 0.45 µm pore size nylon filter paper with and degassed using an ultra-sonic bath before use. The composition and linear gradient is shown in table 2.1.

Table 2.1 Change in mobile phase composition over time

Time (minute)	7.5 or 15 mM TBA in water	7.5 or 15 mM TBA in ACN
0	98%	2%
120	20%	80%

Mobile phase was run for at least one hour to have the column fully equilibrated. A Diode array detector was employed to detect fucose at four different wavelength of 190 nm, 210 nm, 232 nm, and 254 nm.

2.2.6.2 Reversed phase chromatography with protamine sulphate

Mobile phase containing protamine sulphate was used in a reversed phase mode to separate fucose. Stock solution of protamine (with molecular weight of approximately 5 kDa) was used to make mobile phase consisted of 1 µM protamine in water and 1 µM protamine in acetonitrile. Protamine stock solution (125 µL) was dissolved in 250 mL of MilliQ water or acetonitrile to make this solution. Mobile phase was also filtered using 0.45 µm pore size nylon filter paper with and degassed using an ultra-sonic bath before use. A Microsorb C_{18} column (150 mm, 4.6 mm ID) (Varian, CA, USA) was used. A Grace C_{18} guard column was attached to the column's inlet. A Dionex UV-Vis detector was employed to detect fucose and the wavelength of detection was set to 195 nm. Mobile phase was run in linear gradient mode with its change in concentration over time is shown in table 2.2.

Table 2.2 Change in mobile phase composition over time

Time (minute)	1 µM protamine in water	1 µM protamine in ACN
0	95%	5%
15	50%	50%
16	95%	5%
20	95%	5%

2.2.6.3 Reversed phase chromatography with TFA

Mobile phase containing TFA was used in a reversed phase mode to separate fucose. TFA (1 mL) was dissolved in 1000 ml of Milli-Q water or acetonitrile to make a total concentration of 0.1% TFA. Mobile phase was also filtered using 0.45 µm pore size nylon filter paper with and degassed using an ultra-sonic bath before use. A Microsorb C_{18} column (150 mm, 4.6 mm ID) (Varian, CA, USA) was used and a Grace C_{18} guard column was attached to its inlet. Mobile phase was run in a linear gradient mode with its change in concentration over time is shown in table 2.3. A Dionex UV-Vis detector was utilised to detect fucose in the sample and the wavelength of detection was set to 195 nm.

Table 2.3 Changes in mobile phase composition over time

Time (minute)	0.1% TFA in water	0.1% TFA in ACN
0	95%	5%
15	50%	50%
16	95%	5%
20	95%	5%

2.2.6.4 Normal phase chromatography using an amino column (stage 1)

Solutions containing 1 mg/mL of fucose, xylose, fructose, glucose and galactose made in ***2.2.1.1*** was used. In a 15 mL centrifuge tube, a solution containing mixture of the monosaccharides were made with the final concentration of each monosaccharide in the solution was 100 µg/mL. Each monosaccharide solution (10 µL) was injected into a Shodex Asahipak NH2P-50 4E column (250 mm, 4.6 mm ID) (Shodex, Tokyo, Japan) with Asahipak

NH2P-50G 4A guard column (Shodex, Tokyo, Japan) attached to its inlet. The column compartment temperature was set at 30 °C. Mobile phase, consisted of MilliQ water and ACN, was used in a linear gradient mode at a flow rate of 1 mL/minute. Various linear gradient parameters were used as shown in Table 2.4. Mobile phase was also filtered using 0.45 µm pore size nylon filter paper with and degassed using an ultra-sonic bath before use. The samples compartment temperature was set at 10 °C.

Table 2.4 Changes in mobile phase composition over time

Trial	Time (minute)	Water	ACN
Trial 1	0	20%	80%
	6	30%	70%
Trial 2	0	24%	76%
	6	30%	70%
Trial 3	0	24%	76%
	10	30%	70%
Trial 4	0	24%	76%
	10	28%	72%
Trial 5	0	24%	76%
	12	28%	72%
Trial 6	0	24%	76%
	10	32%	68%
Trial 7	0	24%	76%
	15	32%	68%
Trial 8	0	24%	76%
	15	28%	72%
Trial 9	0	24%	76%
	15	26%	74%

Different method was also tried by running mobile phase in isocratic mode with composition of water and ACN shown in table 2.5. Other parameters; flow rate, column temperature, sample temperature and CAD parameters; were kept the same.

Table 2.5 Changes in mobile phase composition over time

Trial	Water	ACN
Trial 1	30%	70%
Trial 2	25%	75%
Trial 3	20%	80%
Trial 4	15%	85%
Trial 5	10%	90%
Trial 6	5%	95%

A charged aerosol detector (CAD) was employed to detect various monosaccharides. The mobile phase is normally converted to small droplets due to the pressure from the nitrogen gas. This pressure was constantly maintained at 35.0±0.1 *psi*. Total gas flow on the CAD was observed at 1.03 when the acquisition was running while flow ratio was at 0.96 and the voltage was set to be at 20.6 Volt. Data acquisition and instrument control were performed using Chromeleon 7 Chromatography Data System.

2.2.6.5 Normal phase chromatography using an amino column (stage 2)

Normal phase chromatography with an amino column in stage 2 used a mixture of ammonium acetate solution and ACN as mobile phase. Other parameters were kept the same to that of the method in ***2.2.5.4***. Ammonium acetate solution was made by dissolving ammonium acetate crystal in MilliQ water to a certain final concentration. The pH of this solution was then adjusted by adding diluted acetic acid (1% v/v) until the expected pH (4.0, 4.75 and 5.0) was achieved. This ammonium acetate solution was then filtered through 0.45 µm pore size nylon filter paper and degassed with ultra-sonic bath before use.

2.2.6.6 Optimisation of the method candidate

Fucose-spiked plasma sample was prepared using a method described in **2.2.1.3.** Several parameters of the chromatography were changed to obtain a method which give optimum

performance in terms of peaks separation and intensity which are depicted by several chromatographic parameters namely: peak intensity, resolution, asymmetry and theoretical plate number. These included the use of different composition of ammonium acetate and ACN, different pH of the ammonium acetate solution, different strength of the ammonium acetate and also the filter and power function value in CAD.

Mobile phase containing ammonium acetate and ACN with the percentage composition of 30:70, 25:75, 20:80, 15:85 or 10:90 was used. Ammonium acetate with the concentration of 2.5, 5, 10, 25 or 50 mM was used. Various pH of ammonium acetate of 4.0, 4.75 or5.0 was also used to determine the influence of the pH to the analysis. Changes were also made to the filter and power function value of the charged aerosol detector to fine tune the chromatogram. The filter setting was set to 3, 5 or 7, while the power function value of the CAD was changed to 1.0, 1.5 or 2.0.

2.2.7 Assay performance

The performance of the method was validated according to Q2 (R1) guideline: Validation of Analytical procedure from International Conference of Harmonisation (ICH). This included determination of the method's specificity, linearity, accuracy, precision, and robustness.

2.2.7.1 Specificity

Specificity of the developed method was determined by analysing four different types of samples: fucose standard solution in water containing 25 µg/mL of fucose, blank plasma, blank plasma spiked with fucose solution containing 25 µg/mL of fucose and blank plasma spiked with fucoidan solution containing 200 µg/mL of fucoidan.

2.2.7.2 Linearity

Different concentration of fucose solutions (1-80 µg/mL) were used to determine the relationship between fucose concentration and CAD response. Calibration curve was made by plotting fucose concentration and peak area to generate equation formula and regression coefficient.

2.2.7.3 Accuracy, precision and reproducibility

Different solutions of fucose with low, medium and high concentration were used to determine the accuracy, precision and reproducibility of the developed method. Regression equation was used to determine the concentration of fucose based on the detected area. Intra- and inter-day accuracy value (n=6) for each concentration were calculated with the formula:

$$\frac{C_1}{C_0} x\ 100$$

where C_0 is the expected concentration and C_1 is the obtained concentration of the sample. Intra- and inter-day (5 consecutive days) precision was determined using peak areas by repeated analysis of each samples (n=6). Reproducibility was investigated by determining mean intra- and inter-day (5 consecutive days) peak retention time of each analyte (n=6).

2.2.7.4 Robustness

The robustness was investigated by repeated injection of fucose standard (n=6, 50 µg/mL) and slightly altering four of the chromatographic parameters; the concentration of ammonium acetate (4.5 or 5.5), the pH value of ammonium acetate (4, 4.75 or 5), flow rate of the mobile phase (0.95 to 1.05 ml/minute) and the temperature of the column (28 to 32°C).

2.2.7.5 Stability

Blank plasma (n=5) were spiked with 50 µg/mL of fucose. Samples were subjected to protein precipitation using acetonitrile. Protein precipitate was then underwent digestion with 20% TFA. The supernatant obtained after centrifugation was then subjected to HPLC analysis (Day 0) or stored at -20°C for up to a period of 31 days. The stored samples were withdrawn after 7, 14, 21 and 31 days and thawed at room temperature before HPLC analysis. The peak area of each analyte was compared to that obtained on the initial day of analysis (Day 0).

2.2.7.6 Extraction recovery

Recovery of fucoidan and fucose in plasma was investigated by comparing the concentration of the spiked fucose in blank plasma after precipitation using acetonitrile as described in ***2.2.3.4*** to that dissolved in water. Recovery was calculated with the formula

$$\frac{C_p}{C_w} x\ 100$$

where C_p is the concentration of fucose in blank plasma and Cw is the concentration of fucose in water.

2.2.8 Patients

2.2.8.1 Recruitment

Patients were recruited based on inclusion and exclusion criteria listed in table 2.5. Recruitment was performed in accordance with the National Health and Medical Research Council (NHMRC) National Statement on Ethical Conduct in Human Research (Commonwealth of Australia, 2007), the 18th World Medical Assembly (Helsinki 1964) and amendments at subsequent World Medical Assembly. Identification of the patients was done by the participating oncologist, surgeons or health care workers. Certain patients-related information

were recorded. These included trial code; patient's initials and hospital number (URN); and completed eligibility checklist. These information were retrieved from the patient registration log which were created by the participating treatment centre.

Table 2.6 Inclusion and exclusion criteria for patient recruitment

Inclusion criteria	1.	Patients have been diagnosed to have active malignancy of either organ tumour or lymphoma
	2.	Patients also were suitable candidate for active treatment for their malignancy with either chemotherapy or hormonal therapy which will be determined by their treating oncologist
	3.	Patients were 18 years old or older
	4.	Written informed consent have been given to all the patients prior to the recruitment
	5.	Patients have the ability to swallow capsule
	6.	Patients were assured to be accessible for treatment and follow-up. Patients randomised in the study were also available for complete documentation of the treatment, adverse reactions and follow up.
	7.	Patients have completed 1 cycle of chemotherapy or 4 weeks of hormonal therapy
	8.	Patients also had at least 2 cycles of chemotherapy or 3 weeks of hormonal therapy remaining in their planned treatment course
Exclusion criteria	1.	Patients reluctant or unable to cease other CAM (including traditional Chinese medicine, other herbal, homeopathic or naturopathic medicine) at least week prior to trial commencement
	2.	ECOG Performance status of ≥ 3
	3.	Life expectancy of less than 12 weeks
	4.	Inadequate haematopoietic (WBC < 3.0 x 109/L; ANC < 1.5 x 109/L, platelet < 100 x 109/dL), or renal function (GFR < 50 mL/min)
	5.	Inadequate hepatic function (either AST/ALT > 2.5 x ULN, or > 5 x ULN in case of liver metastases, or bilirubin > 1.5 x ULN)
	6.	Pregnant or lactating women
	7.	Cerebral or leptomeningeal metastases that are unstable in spite of appropriate therapy. Serious inter-current medical illness including (but not restricted to) HIV, active infection, unstable angina, severe heart failure, or ongoing surgical complications
	8.	Major surgery within 2 weeks prior to study commencement
	9.	Concurrent Radiotherapy

Exclusion criteria (continue-d…)	10.	Clinical evidence of current or impending bowel obstruction
	11.	Documented allergy to the particular CAM
	12.	Concurrent warfarin therapy
	13.	Patients must not be participating in (or planning to participate in) trials of other pharmacological agents during their time on this study

Patients were receiving Docexatel with a maximum dose of between 75 and 100 mg/m^2 every 3 weeks as a single agent or undergoing hormonal treatment using Anastrozole 1 mg daily. Beside the chemotherapy or hormonal treatment, the patients were also given capsule containing *Undaria pinnatifida* derived fucoidan extract manufactured by Marinova Pty Ltd. Each patient were given 250 mg capsule containing 187.5 mg fucoidan four times a day; thus total of 750 mg fucoidan were given. Capsule were self-administered orally by the patients after food in consecutive days for the total of three weeks.

2.2.8.2 Sample collection and storage

Blood was collected at the same time of routine blood test to avoid additional venepuncture. Part of this blood sample was used immediately as the standard of care. Patients' blood was collected prior to and after the administration of fucoidan. Other part of the blood sample for the determination of fucose level was stored in a freezer under the temperature of -70 °C and was then used once the analytical method has been developed.

2.2.8.3 Patients' plasma sample preparation

Patients' plasma (500 µL) was pipetted and transferred into a 15 mL polypropylene centrifuge tube. Into this tube, 4 mL of acetonitrile was added. The mixture was then vortex mixed for 1 minute and centrifuged at 3500 rpm for 20 minutes. The supernatant was carefully taken and discarded while the precipitate was kept intact. Into the tube containing precipitate, 20% TFA (3 mL) was added and then vortex mixed for 1 minute. The tube's cap was then tightly closed

and sealed with seven to eight layers of paraffin film. Subsequently, the tube was kept on a heating block and heated at 100 ℃ for 6 hours with vortexed mixing every 1 hour. Then, it was kept on open bench until it reached room temperature. A vacuum evaporator was then used to evaporate the solution to dryness. Milli-Q water (1 mL) was then added and vortex mixed until the dried sample was completely dissolved. The mixture was then subjected to centrifugation at 3500 rpm for 20 minutes. Finally, the supernatant was carefully taken and subjected to HPLC analysis using the developed and validated method. The patients' plasma sample was prepared and analysed once for each sample.

Chapter 3 – Result and Discussion

The availability of an analytical method as a tool to reach the goal of this study, which is to determine whether or not fucoidan is getting absorbed after oral administration, is vital. It has been mentioned earlier that the method used by Irhimeh and Tokita has, to our opinion, several drawbacks. Therefore, different method and approach was chosen in this study. High Performance Liquid Chromatography (HPLC) method was used instead of the same ELISA method, which was also based on the following several consideration.

First, there is uncertainty on the absorption of fucoidan, whether it will be absorbed as a whole molecule or undergo degradation in the GIT prior to the absorption, if it is absorbed at all. Hence, developing an HPLC method to detect fucoidan as a whole molecule was not considered as a feasible approach. This was also due to the fact that fucoidan has very high molecular weight. Resolving such a big molecule would have been tedious and time consuming without a guarantee that the existing compound in the subject's plasma sample would be the same to that of the analyte used in the development stage. This would have also caused difficulty in identification of the analyte of interest due to the variety in the structure of fucoidan.

Second, fucoidan consists of considerably high percentage of fucose in its structure. Fucose is a monosaccharide, the simplest form of sugar. It has exact structure, molecular weight and other identifiable chemical properties which are valuable in identification of a compound using an analytical method. Hence, detecting fucose was thought to be a more probable approach though it was considered as an indirect determination of fucoidan. To do this, several digestion methods to depolymerise fucoidan into fucose was tried. The developed chromatography method was also aimed to detect fucose as the analyte of interest.

Third, the sample used in this study was human plasma which was obtained from subjects who have been given oral fucoidan. Fucoidan in this plasma sample was then digested into fucose. Due to the fact that endogenous fucose also exist in the body, it possibly added to the concentration of fucose from fucoidan. Hence, the amount of endogenous fucose was taken into consideration. Elevating fucose concentration in plasma compared to its initial concentration could probably resulted from the absorbed fucoidan.

Fourth, analytical method using HPLC give broad choices of approach that can be taken. Normal phase, reversed phase, ion-pairing and ion-exchange chromatography are some of the possible approach in liquid chromatography which can be tried to resolve fucose in plasma. These includes but not limited to utilisation of different mobile phase and its composition, column and also detector. Each of these chromatography methods has also been tried in this study with different given result. One that gave the best result in resolving fucose was taken.

Fifth, fucose is a sugar which its structure lack of chromophore and this limit the choice of detector to be used in this study. Though it can still be detected with UV detector when the sample solution contain high concentration of fucose, as it has been tried in this study and will be shown later in this chapter, still it was not considered as the best approach for several reason: the detection should be set at low wavelength which give high noises, the expected fucose concentration in plasma was very low and by increasing the amount of plasma sample to increase the fucose concentration will also increase other compounds concentration from the plasma which will interfere fucose peak. Therefore, in this study we employed Charged Aerosol Detector which has the ability to detect molecule based on the charged created on the molecule and is proportional to the quantity of the analyte present. The requirement that the

analyte should be non-volatile or at least semi-volatile was fulfilled since fucose is considered non-volatile due to its high vapor pressure.

The above mentioned consideration was regarded as guideline in the method development in this study and will be discussed in this chapter. This study was started with the initial trial in obtaining appropriate chromatography method to elute fucose standard solution in water. Once this had been achieved, we continued to test the method on fucose-spiked plasma to obtain appropriate method to analyse fucose in plasma. Subsequently, several digestion methods were then tried to fractionate fucoidan which was spiked into plasma. The effectivity of the digestion method was determined by subjecting plasma containing the digested fucoidan with the method developed to analyse fucose-spiked plasma. Several extraction methods were also tried to retrieved fucose from the digested fucoidan. Finally, once the appropriate methods to digest fucoidan, extract fucose from plasma and analyse fucose in plasma sample had been obtained and developed; those methods were then applied to analyse fucose concentration in plasma sample containing fucoidan.

The orally administered fucoidan used in this study was extracted from brown seaweed *Undaria pinnatifida*, brand Maritech®, and was obtained from Marinova. Since this was a commercially marketed product, its characteristic (the amount of fucose and other monosaccharides, the composition of sulphate group and its purity) has been well determined. This well characterized fucoidan assure similarity of the orally given fucoidan to each patient. Hence, different figure shown by the patients, either in its investigated biological effect or its absorption following oral administration, can probably due to different response from each patient to the given fucoidan. The composition of compounds exist in the Maritech® *Undaria pinnatifida* fucoidan are listed in table 3.1.

Table 3.1 The characteristic of the Maritech® *Undaria pinnatifida* fucoidan[63]

Components	Percentage composition (% w/w)
Fucoidan	≥75.0
Fucose	22.0
Galactose	21.2
Mannose	0.9
Glucose	0.9
Uronic acid	4.3
Sulphate	25.2

Undaria pinnatifida derived fucoidan, as well as fucoidan extracted from different seaweeds, has been studied for its bioactivities such as anticancer and anticoagulant properties. These biological properties was investigated not only in vitro but also in vivo. The result of these studies, particularly those obtained through in vivo investigation, are useful as they usually provide information about the required dose of tested fucoidan to significantly show the investigated effect. Hence, by using the same Undaria pinnatifida fucoidan, a relation can be made between the results obtained in our study to the study investigating its bioactivities. The amount of fucoidan absorbed after oral administration can then be compared to the dose of fucoidan required to promote particular investigated bioactivities.

3.1 Ion-pair reversed phase chromatography

Reversed phase method in liquid chromatography has been extensively used for the separation of a wide range of pharmaceutical molecules[88]. While it has been mostly utilised for the separation of non-polar molecules, the newer types of reversed phase columns have the ability to resolve polar compounds as well[88]. With increasing purity of available stationary phases, the reversed phase columns are now not only used for acidic molecules but also utilised for basic compounds[88-90]. Moreover, keeping the basic principle of reversed phase chromatography which uses non-polar column; tweaking around the method by, for example, adding ionic

molecule to the mobile phase had created the new term of ion-pairing chromatography with which ionic molecules are mostly resolved through liquid chromatography.

Several studies have successfully resolved various types of carbohydrate molecules (e.g. heparin and heparan sulphate) using an ion-pairing reversed phase chromatography[91-93]. Therefore, we also tried the reversed-phase chromatography in the early stage of this study. Fucose, as other sugars, is a polar compound, making it difficult to retain and resolve on a C_{18} column in the absence of an ion pairing reagent. Therefore, two different types of ion pairing reagents were investigated to determine their ion pair capacity and, hence, the effect on chromatographic separation.

3.1.1 Effect of the ion pair reagents

The effect of TBA on the separation of fucose was investigated in the concentration range of 7.5 to 15 mM. The mobile phase was composed of TBA (7.5 mM) in water (mobile phase A) and TBA (7.5 mM) in acetonitrile (ACN) (mobile phase B). It was attempted to determine the optimal concentration of ACN for 7.5 mM TBA by steadily increasing the concentration of ACN from 2 to 20% over a period of 120 minutes. However, with this approach, fucose did not retain onto the column and was eluted at approximately 2.5 minutes as shown in figure 3.1. At this point, two reasons were thought to be responsible for early elution of fucose: 1) in ion pair reversed phase chromatography, an analyte (e.g. fucose) interacts with the oppositely charged ion pair (e.g. TBA) adsorbed onto the hydrophobic surface of the column and remains there until the concentration of the organic modifier (in this case ACN) is sufficient enough to elute the analyte. Therefore, the initial concentration of ACN (2%) was considered too high and therefore it did not allow fucose to retain onto the column; 2) the resolving power of 7.5 mM TBA was not sufficient enough that would allow its efficient interaction with fucose.

Therefore, the available approach was to further decrease the initial concentration of ACN and/or to increase the concentration of TBA. Hence, the concentration of ACN (containing 15 mM TBA) was steadily increased from 0.5 to 10% over a period of 120 minutes. The retention time of fucose did not change despite altering the concentration of ACN to form a shallow gradient. Then the concentration of TBA was increased from 7.5 to 15 mM with the gradient of ACN from 0.5 to 10% over 120 minutes. Increased in the concentration of TBA also did not influence the retention time of fucose.

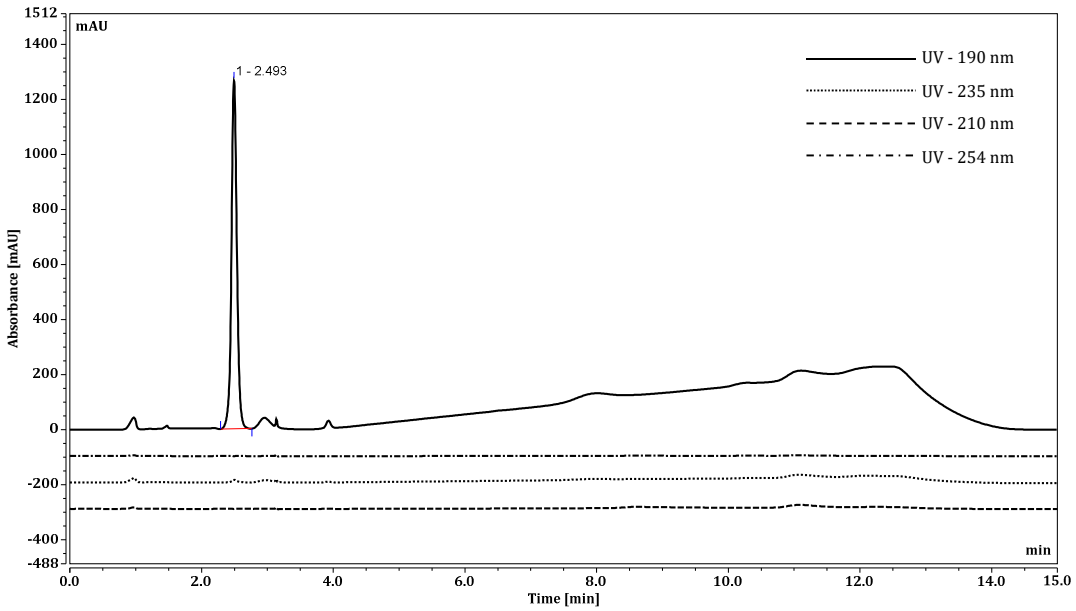

Figure 3.1 RPIP-HPLC separation of fucose. Experimental conditions: Grace Apollo C18 (250_4.6 mm), 5 µm; eluent containing 15 mM TBA in water and ACN; detection wavelength 190, 210, 235 and 254 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

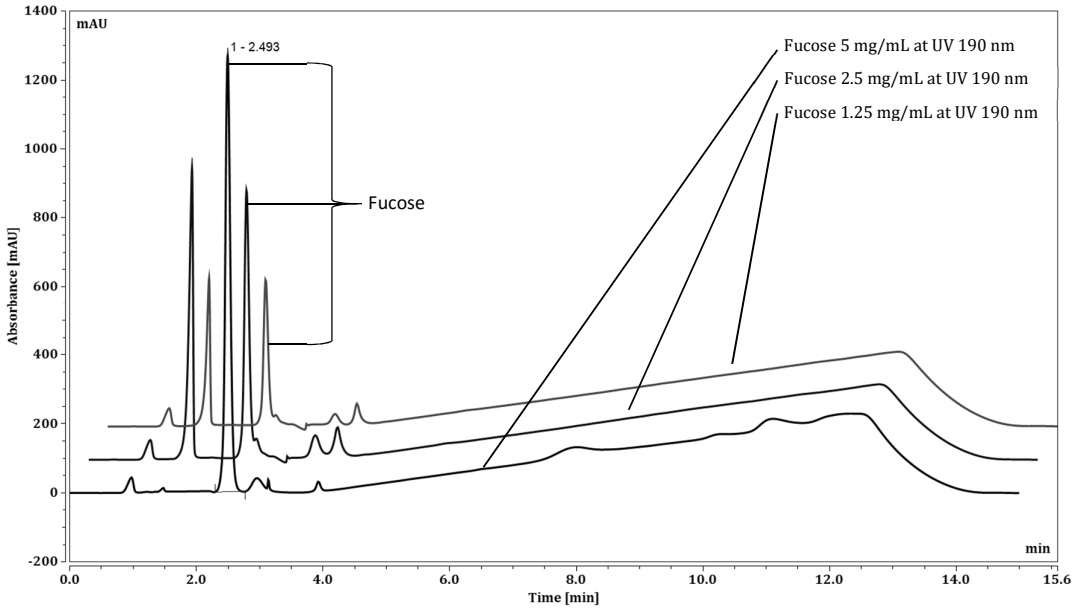

Figure 3.2 RPIP-HPLC separation of fucose solution containing 1.25, 2.5 and 5 mg/mL of fucose. Experimental conditions: Grace Apollo C18 (250_4.6 mm), 5 µm; eluent containing 15 mM TBA in water and ACN; detection wavelength 190 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

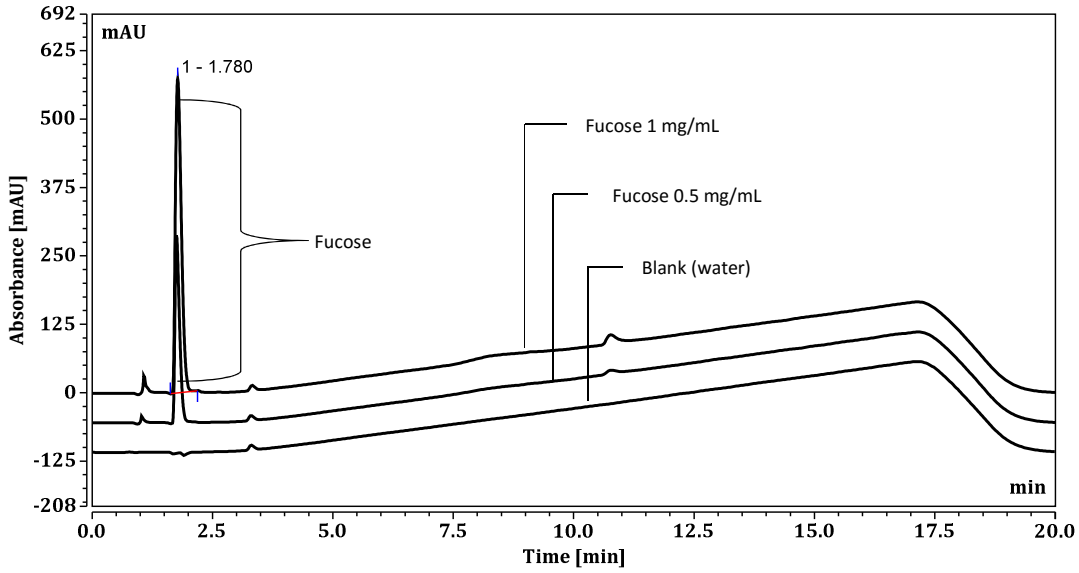

Figure 3.3 RPIP-HPLC separation of blank (MilliQ water) and fucose solution containing 0.5 and 1 mg/mL of fucose. Experimental conditions: Grace Apollo C18 (250_4.6 mm), 5 µm; eluent containing 0.1% TFA in water and ACN; detection wavelength 190 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

Four different UV wavelengths (190, 220, 235, and 254 nm) were used to detect fucose when above mentioned approach was taken. While fucose was detected at 190 nm, no peak was observed on other selected wavelengths (Figure 3.1). Therefore, the separation of fucose in the presence of another type of ion-pairing agent was observed at 190 nm.

The effect of protamine sulphate (1 µM) on the separation of fucose was also investigated. Protamine is a highly positively charged polypeptide which is mainly composed of the amino acid arginine[94]. Therefore, the column was first equilibrated with 1 µM of protamine sulphate to allow its adsorption onto the column. The linear gradient of ACN was changed from 5% to 50% over a period of 15 minutes. However, with this approach fucose did not retain on to the column and eluted at approximately 2 minutes.

The separation of carbohydrates using an ion-pairing reagents (such as TBA, tetra butyl ammonium hydroxide) has been reported before[95]. The analyte analysed in the previous studies were obtained through either chemical or enzymatic digestion of large molecules such as heparin and heparan sulphate. After digestion process, the obtained sugar molecules (often known to as oligosaccharides) had high negative charge attributed to the presence of sulphate groups. For example, total bonded sulphate in a low molecular weight heparin solution after acid hydrolysis is reported to be approximately 39% (w/w)[96]. These highly negatively charged molecules were thought to interact with the positively charged TBA or protamine sulphate resulting in their retention when a reversed phase C_{18} column was employed. Unlike oligosaccharides, fucose (a monosaccharide) does not possess a high net negative charge. Therefore, the ionic interaction between fucose and TBA or protamine sulphate was not strong enough for fucose to retain onto the column even when the initial concentration of organic modifier was as low as 0.5%.

One possible approach to overcome the above mentioned problem was the use of sulphated fucose. The presence of sulphate groups could increase the interaction between fucose and ion pairing reagent and hence delay the elution of fucose. However, the goal of the study was to investigate the concentration of fucose before and after oral administration of commercially available fucoidan. For this purpose, chemical digestion of patients' plasma samples was required to facilitate complete breakdown of fucoidan into fucose. The digestion process results in a complete loss of bonded sulphate groups. Therefore, it was considered crucial to use non-sulphated fucose during the development phase of an analytical method.

3.2 Normal phase chromatography

In normal phase chromatography, the stationary phase of a column is considerably more polar than the mobile phase used for the separation of an analyte. This feature of a normal phase column provides an advantage over reversed phase column when used for the analysis of polar compounds such as monosaccharides and oligosaccharides. In some cases, however, the utilisation of ion-pairing reagent will also improve the ability of reversed-phase chromatography in the analysis of polar compounds. The polar characteristic of the column's stationary phase in a normal phase chromatography improves retention on such polar molecules. A polar analyte will easily be attracted to column's stationary phase with similar polarity. The strength of mobile phase in terms of its polarity will then play a role in the separation of molecules in a sample when subjected to analysis with HPLC. In regards to the analysis of monosaccharides, some columns are, in fact, specially designed for carbohydrate or particularly monosaccharides analysis. One such type of column is Shodex Asahipak NH2P-50. This column is also classified as a HILIC (hydrophilic interaction liquid chromatography) column[97]. It contains amino-bonded silica as the stationary phase. The mobile phases used with

amino-bonded silica columns are usually similar to that of reversed phase chromatography. This type of column not only allows to analyse chemically polar compounds but also those with a net charge in their structure[98].

We initially utilised this column for the separation of fucose using the method suggested by its manufacturer. The mobile phase was consisted of 25% water and 75% ACN. Mobile phase was run in isocratic mode at the flow rate of 0.5 mL/minute. Column temperature was set to 30 ℃. Sample was auto-injected and was kept under 10 ℃ in an auto-sampler compartment. The type of detector was changed from Ultraviolet (UV) detector to Charged Aerosol Detector (CAD). The CAD parameters were set as: Data Collection Rate=100, Nebuliser Temperature=ambient temperature, Power Function=1.0, Filter Value=3.0.

The utilised detector was changed due to the fact that UV detector has limitation in detecting compounds lack UV chromophore, such as carbohydrates. The UV detector used in the current study was still able to detect fucose. However, we needed to use high concentration of fucose (5 mg/mL) as it is shown in Figure 3.4. However, the UV intensity which was depicted by the height of the chromatographic fucose was quite low compared to CAD intensity. The UV detection of an analyte depends on the wavelength selection. Depending on the UV chromophore of an analyte, different wavelengths can give different intensity. The maximum peak intensity can be obtained at a particular wavelength depending on the type of analyte. We tried several wavelengths and found out that the maximum detection for fucose was at 190 nm. At this wavelength, noise from mobile phase was high and therefore it hampered the detection. The detection of fucose using UV detector was difficult when the concentration of fucose was lower than 5 mg/mL. On the other hand, CAD offers universal detection. This means the detection of an analyte depends on the mass as well as the structure of the analyte of interest.

It detects molecules which have been previously charged and the detector's signal intensity was related to the mass of analyte flowing through the detector and not to its UV chromophore.

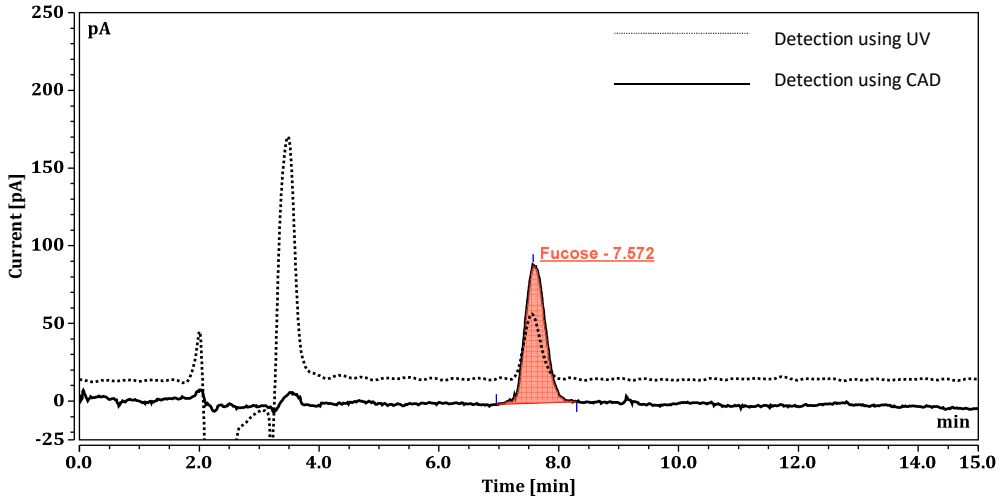

Figure 3.4 Normal phase-HPLC separation of fucose solution containing 5 mg/mL of fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection using CAD and diode array detector at wavelength 190 nm, flow rate 1 mL/minute, injection volume 10 µL.

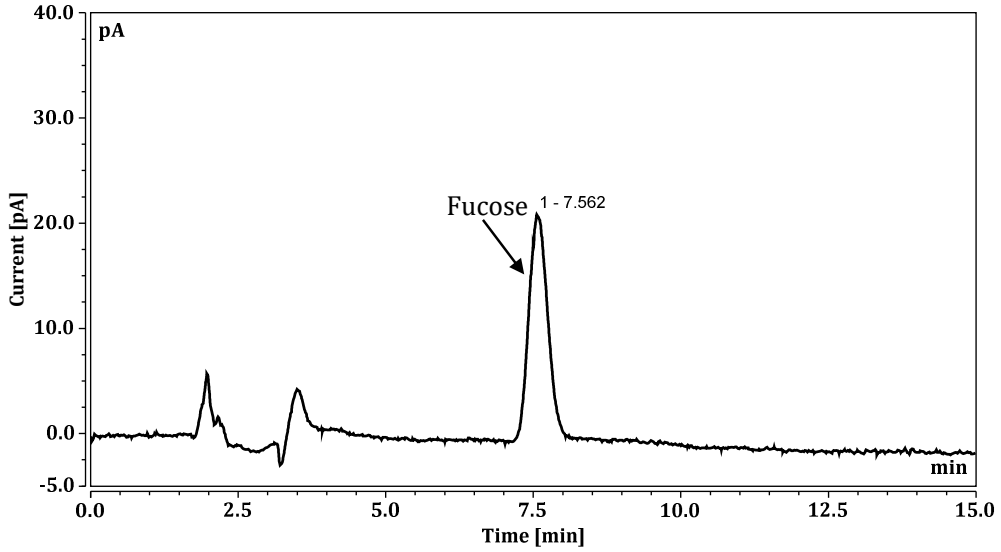

Figure 3.5 Normal phase-HPLC separation of fucose solution containing 5 mg/mL of fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Figure 3.5 shows chromatogram of fucose solution eluted with the above mentioned chromatography parameters. It can be seen that fucose was eluted at 7.5 minutes. This retention time of fucose was considered suitable and therefore we decided to use this column. However, considering the final sample of this study was human plasma which contain endogenous monosaccharides such as glucose, fucose, fructose, xylose and some others; possible interference between fucose and these monosaccharides could occur. Hence, further adjustment to some of the chromatography parameters to obtain the appropriate method was needed. This also to at least help reduce the possibility of the endogenous monosaccharides from interfering fucose peak when the analysis of plasma sample was performed. To do this, mixture of monosaccharides was prepared as described in method ***2.2.1.1***. Solution containing 5 mg/mL of each of these monosaccharides were also prepared. These samples were then subjected to HPLC and were resolved with the previously developed method.

Solution containing each of monosaccharide was injected separately and chromatogram was recorded. Figure 3.6, 3.7, 3.8, 3.9, and 3.10 are chromatograms showing peak of fucose, xylose, fructose, glucose and galactose, respectively.

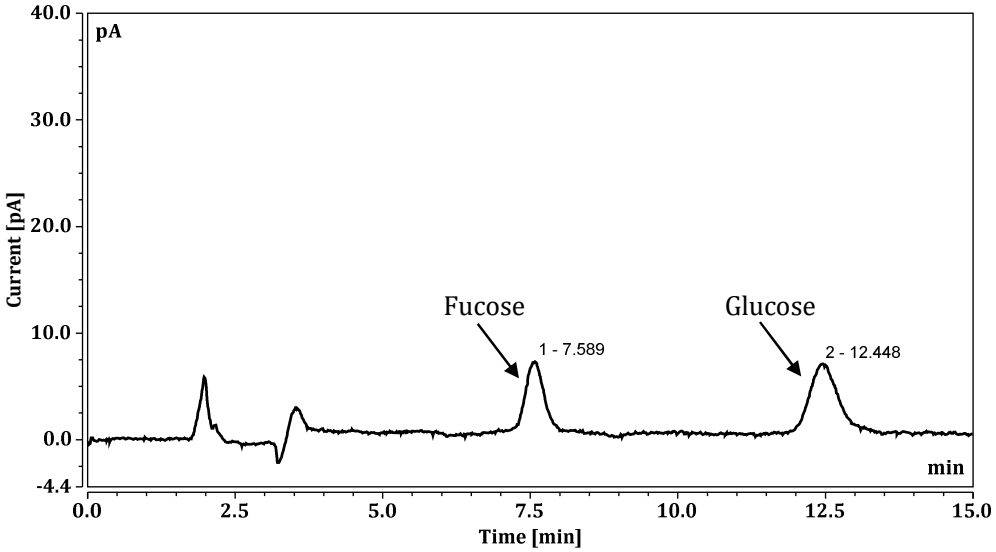

Figure 3.6 Normal phase-HPLC separation of fucose solution containing 5 mg/mL of fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

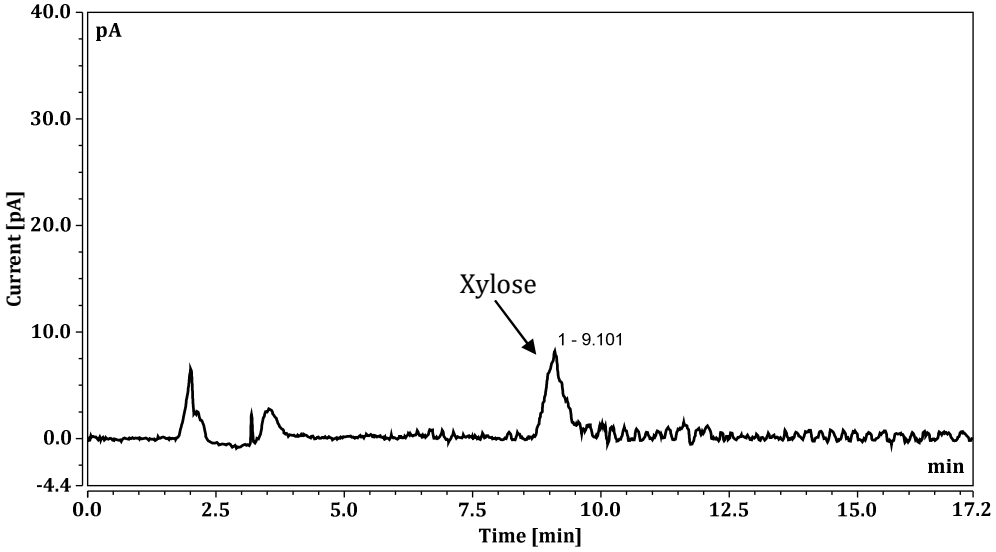

Figure 3.7 Normal phase-HPLC separation of solution containing xylose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

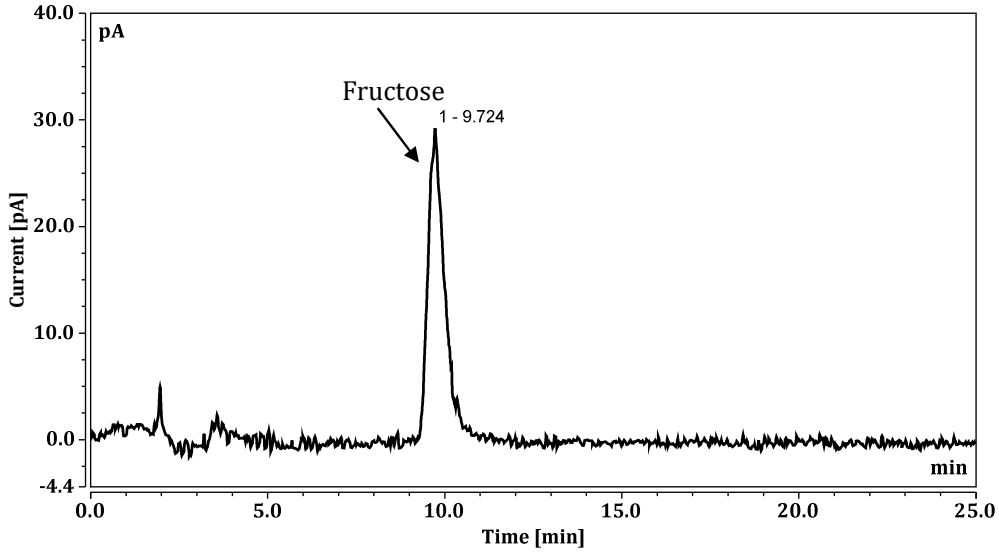

Figure 3.8 Normal phase-HPLC separation of solution containing fructose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

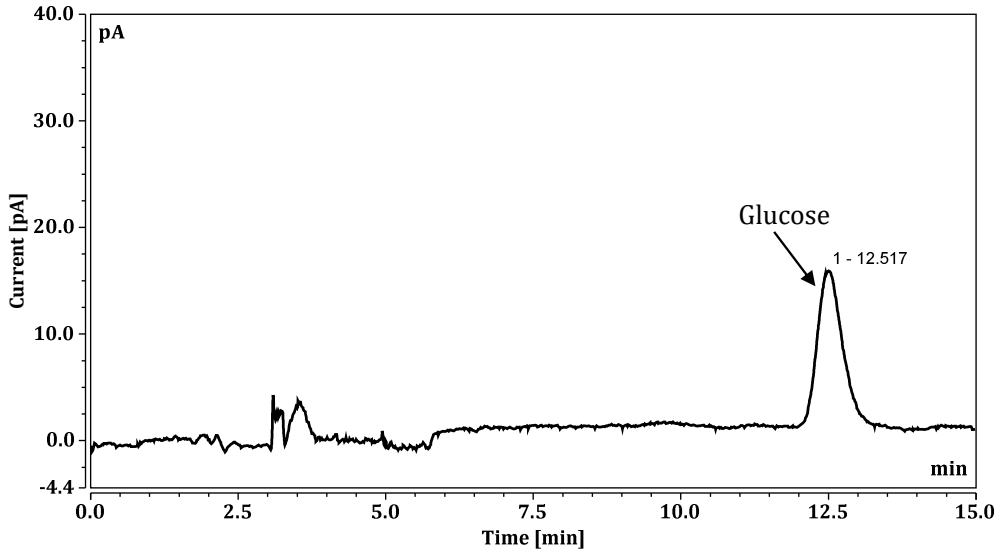

Figure 3.9 Normal phase-HPLC separation of solution containing glucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

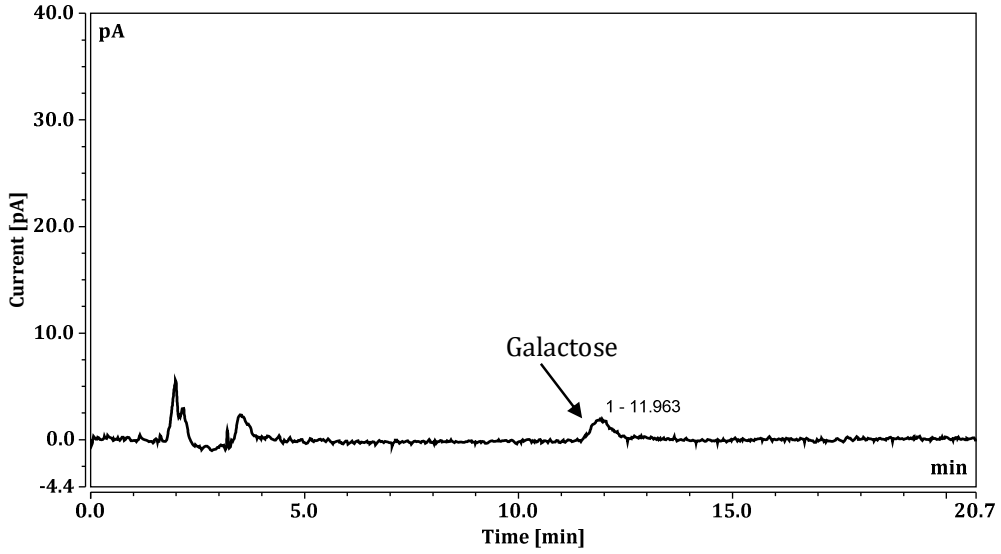

Figure 3.10 Normal phase-HPLC separation of solution containing galactose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

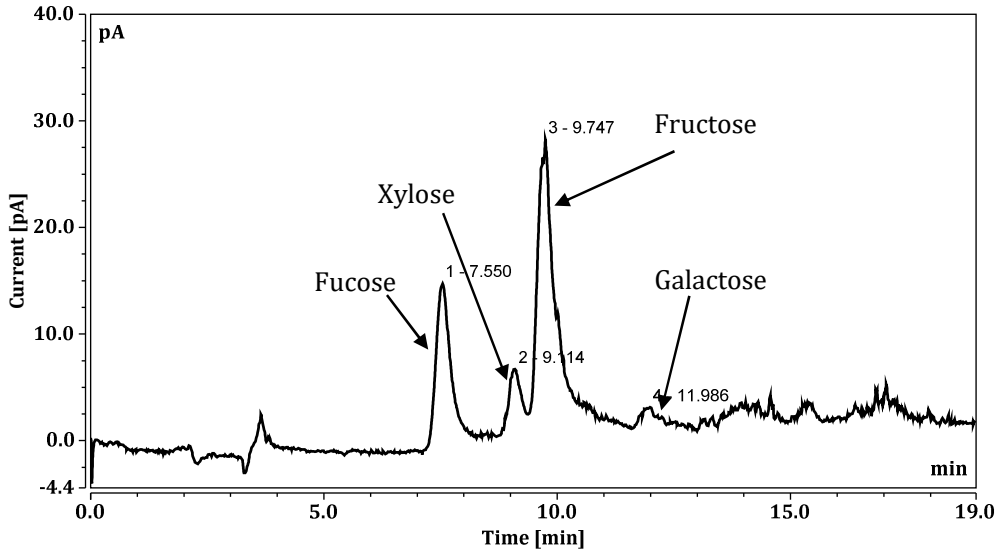

Figure 3.11 Normal phase-HPLC separation of solution containing mixture of monosaccharides. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Figure 3.11 shows the chromatogram of solution containing a mixture of monosaccharides (without glucose) eluted with mobile phase containing 25% water and 75% ACN. The retention time of each monosaccharide was similar to when each of them was analysed separately.

Fucose was eluted first at 7.550 minutes followed by xylose at 9.114 minutes, fructose at 9.747 minutes and galactose at 11.966 minutes. The resolution between fucose and xylose peak, the closest eluted monosaccharide to fucose, was 3.22 which suggested that both monosaccharides were separated nicely. The intense noise was observed when the composition of water in the mobile phase was increased from 20% to 25%. However, this did not occur all the time. We thought, the higher noise was caused by temporary disruption to the CAD nozzle in producing uniform droplets of mobile phase. We then adjusted the mobile phase composition to find out its effect to the monosaccharides separation. Increasing water portion by 3% to 28%, which also changed ACN composition accordingly to make the total composition of 100%, eluted all the monosaccharides faster without changing the order of elution as shown in Figure 3.12. This also increased the peak height of all monosaccharides which also means increasing detection intensity. At 30% of water content in mobile phase, peak height of all monosaccharides was even further increased (Figure 3.13).

The expected goal, by modifying the mobile phase composition, was to obtain a method which gives higher peak intensity from the same amount or concentration of analyte. Such method can possibly offer lower limit of quantification (LOQ) or at least limit of detection (LOD). Unfortunately, the increasing peak height, as a result of the increasing water composition in the mobile phase, did not produce higher peaks resolution between monosaccharides. The resolution between fucose and xylose decreased from 3.22 to 1.75 by 5% increase of water content in mobile phase. This value was above 1.5, the considered as minimum resolution, which suggested the two peaks were still well separated to accurately measure the peak area. However, xylose and fructose were eluted at almost the same time and their peak overlaid to one another. Therefore, we then used different mobile phase composition to obtain better separation between these monosaccharides.

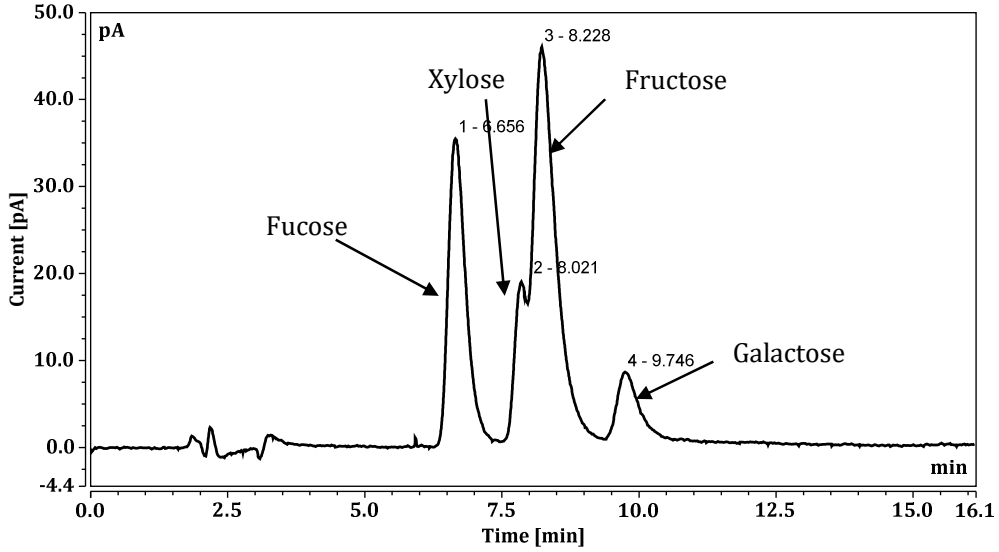

Figure 3.12 Normal phase-HPLC separation of solution containing mixture of monosaccharides. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (28:72); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

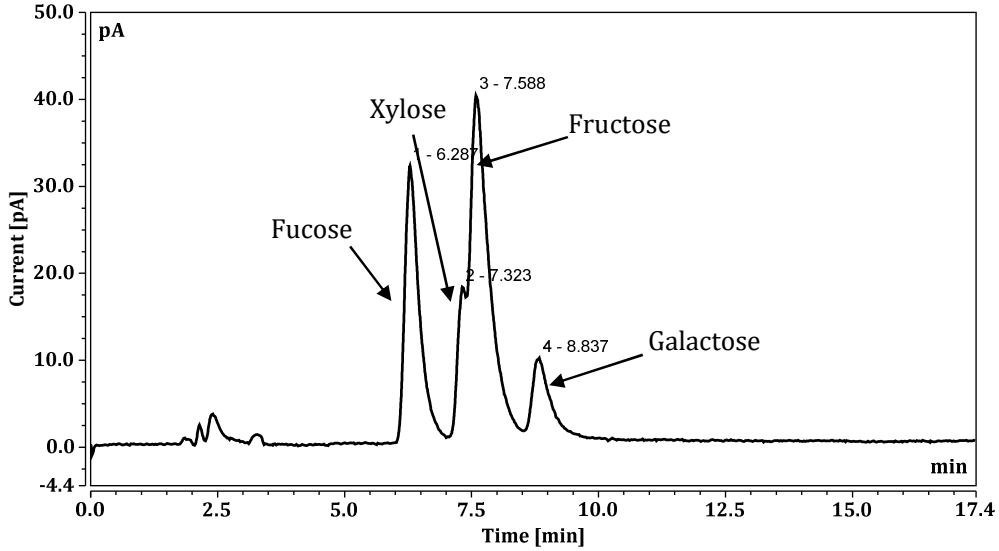

Figure 3.13 Normal phase-HPLC separation of solution containing mixture of monosaccharides. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (30:70); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Different composition of mobile phase containing water in chamber A and ACN in chamber B running in gradient mode as is shown in Table 2.4 was tried. Altering the composition of water

in mobile phase from 20% to 30% in 6 minutes (Trial 1), with the flow rate of 1.0 mL/minute, eluted fucose at 7.8 minutes. Xylose and fructose were eluted afterwards and were still interfering with each another (Figure 3.13). Linearly changed the composition water from 24% to 30% in 6 minutes (in Trial 2) or 10 minutes (in Trial 3) provided no significant difference to that of Trial 1. Similarly, all attempts in Trial 4 to Trial 9 did not produce the expected separation of monosaccharaides. Running mobile phase in gradient mode also created slope in the chromatogram, which sometimes created difficulty in area integration. More time was also needed to re-equilibrate the column. Thus, in the next attempt we decreased the composition of water and run the elution in isocratic mode.

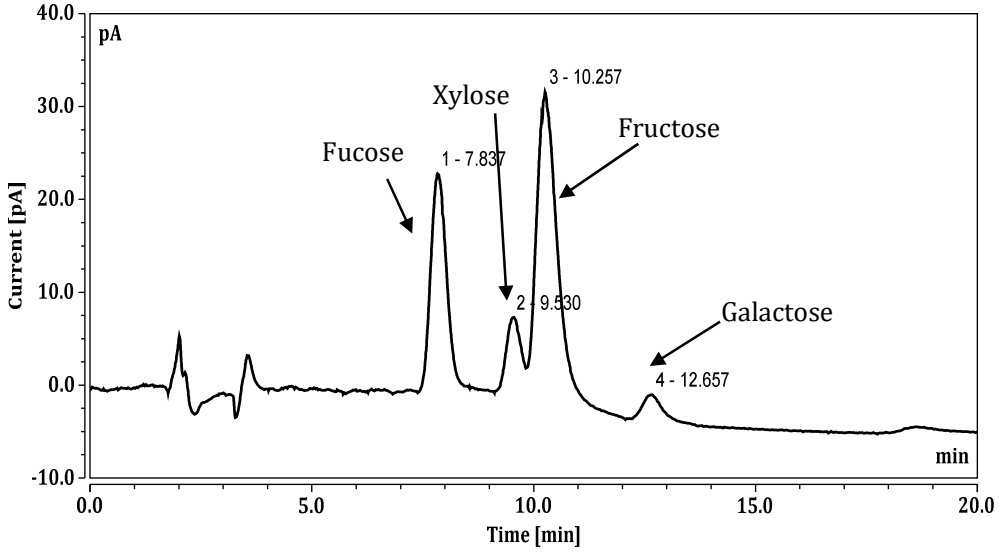

Figure 3.14 Normal phase-HPLC separation of solution containing mixture of monosaccharides. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (water was linearly changed from 20% to 30%); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Mobile phase consisted of 20% water and 80% ACN was eluted the monosaccharides mixture and was run in isocratic mode at the flow rate of 1 mL/minute. Other parameters were kept the same. It appeared that, compared to the previous results, the monosaccharides were well

resolved, particularly those between xylose and fructose (Figure 3.15). Fucose retention time was not significantly changed, at around 7.5 minutes which was considerably appropriate elution time for plasma analysis. This method was thought to be a suitable method to be used for preliminary test on fucose-spiked plasma and will subsequently called as the **method candidate** in this chapter. This preliminary test was intended to determine the suitability of the method when it is used to detect fucose in plasma, prior to investigating its application using patients' plasma samples. Relying on the results obtained through analysis of fucose standard solution was not enough due to the presence of various compounds in plasma. These compounds could potentially interfere fucose which could then hamper the detection of fucose. Therefore, adjustment to the method, when needed, was performed until a suitable method was obtained.

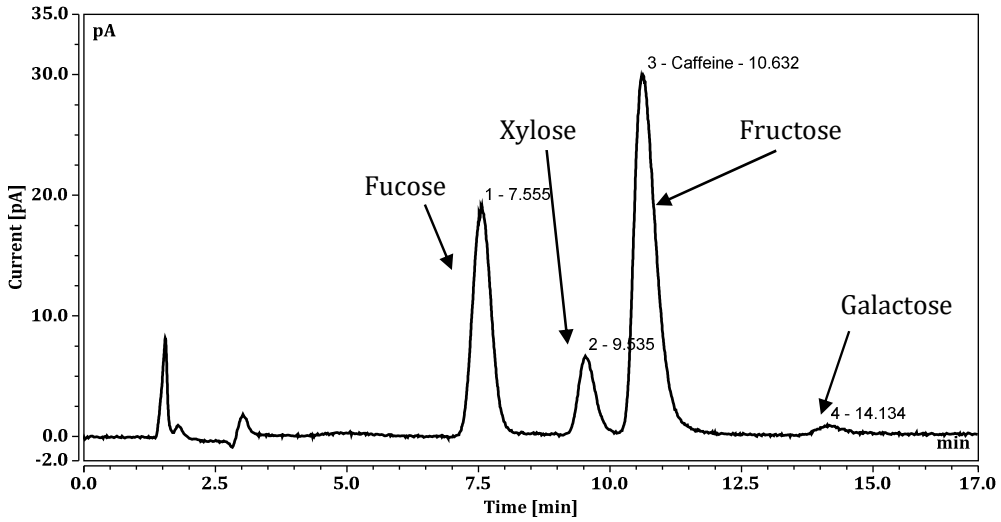

Figure 3.15 Normal phase-HPLC separation of solution containing mixture of monosaccharides. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Another important thing to consider in the analysis of plasma samples using HPLC was the existence of plasma proteins. Plasma proteins not only can disturb the elution of fucose but, if

not removed properly, can also decrease the column's life time and possibly block HPLC compartment such as HPLC flow lines or UV flow cells. On CAD, plasma protein can block the nozzle leads to increasing pressure and disturb conversion of mobile phase into droplets. Hence, several protein precipitation (PPT) methods were tried and its efficacy in removing plasma proteins was tested with the HPLC method suitable for preliminary test. Recovery test of the utilised PPT method was also investigated. This to ensure that the method capable of not only in removing plasma protein but also in preserving fucose in the plasma sample or retrieving fucose from plasma, which depends on the type of the method utilised.

3.3 Plasma protein precipitation Methods

3.3.1 Plasma Protein Precipitation using Ammonium sulphate saturated solution

We used ammonium sulphate saturated solution (ASSS) to precipitate plasma proteins and the method is described under 2.2.3.1 ***Precipitation using ammonium sulphate***. Ammonium sulphate saturated solution has been used to precipitate plasma protein in several studies[99-101]. We investigated the optimum volume needed to precipitate plasma protein by mixing different volumes of ASSS with one part volume of the blank plasma (Table 3.2).

Table 3.2 Precipitation of blank plasma proteins using different volumes of ammonium sulphate saturated solution (ASSS).

Plasma (part volume)	ASSS (part volume)	Result
1	1	Clear solution
1	2	Clear solution
1	3	Clear solution
1	4	Clear solution
1	5	Clear solution

We found out that 1 part or equal volume of ASSS was sufficient to precipitate proteins evident by the appearance of clear final solution at the top after precipitation procedure was applied.

Plasma proteins were observed to be precipitated at the bottom of the test tube. Up to this stage, this method was then tested to precipitate plasma proteins. The supernatant obtained after precipitation using ASSS was subjected to HPLC analysis using the method candidate. The first injection gave broad peaks started from 2 to 8 minutes (Figure 3.16). Since the retention time of fucose using this method was at around 7.5 minutes, this suggested that the endogenous substances would interfere with the elution of fucose.

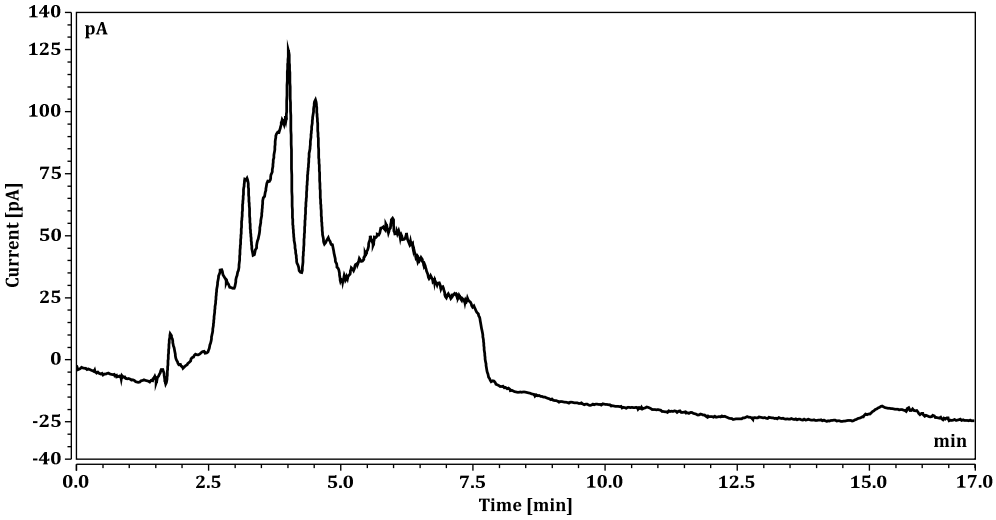

Figure 3.16 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using an equal volume of ammonium sulphate saturated solution (ASSS). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

We then injected ASSS solution containing equal amount of ASSS to that present in the plasma sample to investigate the source of those big peaks eluted from approximately 2 minutes to 7.5 minutes. It can be seen from Figure 3.17, that though quite broad peaks were obtained, those were smaller compared to the chromatogram of the supernatant obtained after precipitation using ASSS as described above. The chromatogram showed peaks started from 2.5 to around 5 minutes which was far enough from possible eluted fucose peak. Spikes were observed on the chromatogram. We thought that the observed spikes were caused by dirty nebuliser nozzle

in CAD due to the injection of plasma. Thus, mobile phase was pumped for approximately an hour until no spikes were observed and straight baseline was obtained.

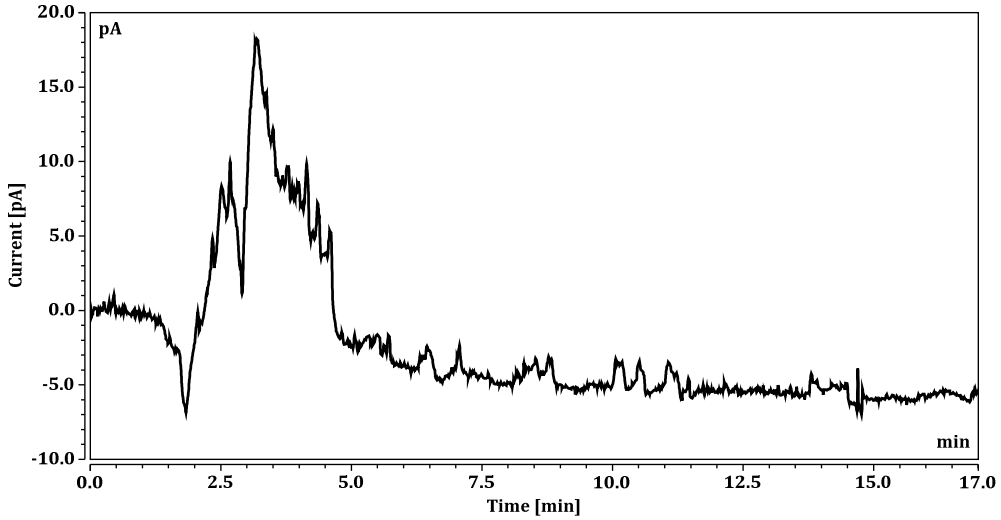

Figure 3.17 Normal phase-HPLC separation of ammonium sulphate saturated solution (ASSS). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

The supernatant taken from the same sample was re-injected to confirm whether the early eluted broad peaks come from the plasma or due to the existence of ASSS. We initially thought to make necessary adjustment to the method to improve separation if the result was repeatable. However, broader peaks were again observed. Unlike in the first injection, this time the compounds were eluted from 2 to 14 minutes (Figure 3.18). Another injection of the same sample also showed similar results. We at this stage thought that some portion of the sample was retained in the column and this part of the sample was eluted in the next sample run. This was probably caused by poor solubility of the retained compounds in the mobile phase or they were strongly attached to the stationary phase in the column, hence mobile phase was not strong enough to elute all the compounds. Another possible cause was the incompatibility of ammonium sulphate as the precipitating agent with the column. Despite the clear appearance

observed following precipitation with ASSS, there was probably some portion of this precipitating agent dissolved in the supernatant. This can be seen by noisy and inconsistent baseline observed in the chromatogram above (Figure 3.17).

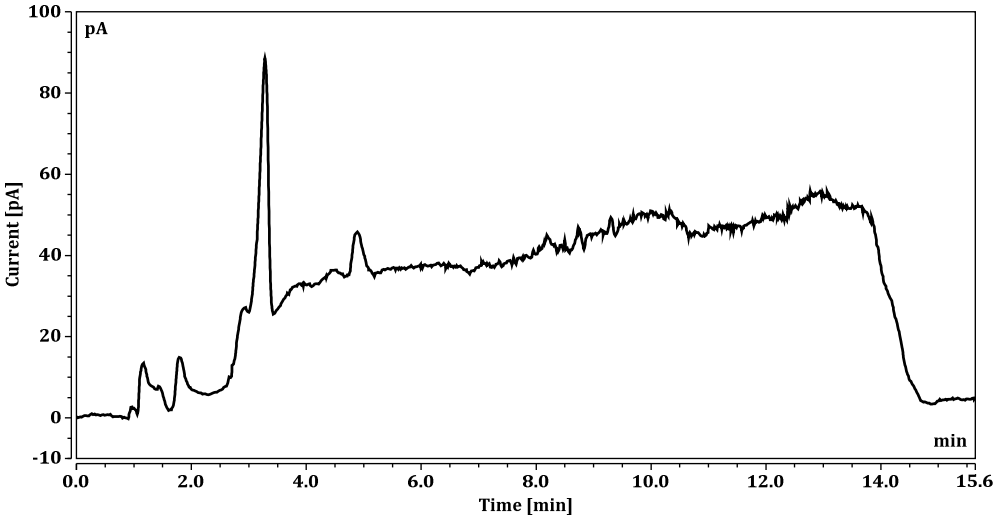

Figure 3.18 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using an equal volume of ammonium sulphate saturated solution (repeated injection). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

To investigate the cause of this problem, a solubility test was performed. This test was conducted by mixing several solutions: fucose solution in water (FS), ammonium sulphate saturated solution (ASSS), water (W) and acetonitrile (ACN)] with each other and the results are shown in Table 3.3. Since the mobile phase consisted of mostly ACN, poor solubility in ACN could have prevented either fucose or ammonium sulphate from ASSS to be completely eluted from the column. This with regards to the possibility that ASSS could be present in the supernatant. Hence, the aim of this test was to determine the solubility of the compounds present in the sample with the mobile phase and the miscibility of one another.

Table 3.3 Solubility test involving fucose solution, mobile phase and ammonium sulphate saturated solution

Test	Mixed solution	Result	Appearance
Test 1	FS + ASSS	Immiscible	Hazy solution was obtained
Test 2	FS + ACN	Miscible	Clear final solution
Test 3	W + ASSS + ACN	Immiscible	Three layers of immiscible solutions were obtained
Test 4	FS + W + ACN	Miscible	Clear final solution
Test 5	FS + ASSS + ACN	Immiscible	Four layers of immiscible solutions were obtained

It can be seen from the table that whenever ASSS was mixed with either fucose solution or ACN, immiscible solution was obtained. On the other hand, a mixture containing fucose solution, water and ACN showed miscible solution. This suggested that ASSS was not miscible with the mobile phase utilised in the method. This suggested that ASSS could potentially precipitated in either water or ACN or both at some extent. Hence, it would have caused retention of ammonium sulphate and it remained in the column for sometimes. Because of this, we concluded that ASSS was not a suitable agent to precipitate plasma protein if the same chromatographic method was used. Therefore, another type of precipitating agent was tested.

3.3.2 Plasma protein precipitation using 95% ethanol

Ethanol, beside ammonium sulphate, had been reported to be useful for precipitation of plasma proteins. The precipitation of plasma proteins was carried out as described under ***2.2.3.2 Precipitation using ethanol***. We tried two different approaches of plasma protein precipitation using ethanol. The first method was a modification of a method previously described by Subhash *et al.* [83]. The second one was a replication of what was described by Jianguo *et al.*[84].

In the first method, we initially determined the optimum volume of 95% ethanol needed to precipitate plasma proteins (Table 3.4).

Table 3.4 Blank plasma protein precipitation using different volume of 95% ethanol

Test	Step 1		Step 2		Result
	Plasma	95% Ethanol	Supernatant	95% Ethanol	
Solution A	100 µL	300 µL	200 µL	200 µL	Hazy solution
Solution B	100 µL	350 µL	200 µL	200 µL	Slight-hazy solution
Solution C	100 µL	400 µL	200 µL	200 µL	Clear solution

In the first step, blank plasma (100 µL) was taken and then 300 (solution A), 350 (solution B), or 400 µL (solution C) of 95% ethanol was added. In the second step, the supernatant (200 µL) was taken and then an equal volume of 95% of ethanol was added. A hazy or slightly hazy solution was obtained when supernatant was taken from solution A or solution B. However, a clear solution was obtained after addition of 400 µL of 95% ethanol to the supernatant taken from solution C. Therefore, it was considered that four times volume of 95% ethanol was sufficient to completely precipitate proteins present in plasma samples. This suggested that the addition of 300 µL and 350 µL of 95% ethanol was not enough to completely precipitate protein from 100 µL plasma. Hence, four times volume of 95% ethanol was considered as the optimum volume to precipitate plasma protein and was tried in this study. The supernatant obtained from this method was then subjected to the HPLC analysis using the method candidate described above (sub chapter 3.2).

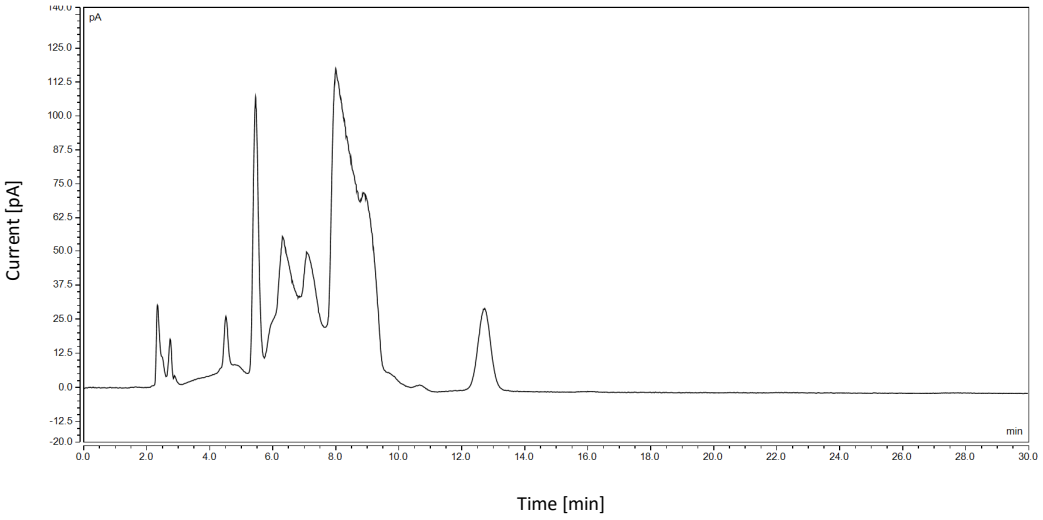

Figure 3.19 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using four times volume of 95% ethanol. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

Figure 3.19 shows the chromatogram of supernatant obtained after precipitation of plasma proteins using four times volume of 95% ethanol. It is apparent from the chromatogram that endogenous compounds from blank plasma were still observed in the supernatant and could have disturbed elution of fucose. Similar to that of the chromatogram obtained after precipitation using ASSS, continuously eluted endogenous compounds were observed from 2 minutes to around 10 minutes. Continuously eluted compounds were also detected in the supernatant following precipitation using cold ethanol (Figure 3.20). Two peaks from unknown compounds were detected at around the expected retention time of fucose. This will similarly hinder the detection of fucose in the injected supernatant. Hence, we decided to again use different precipitating agent. This time we used 80% ACN.

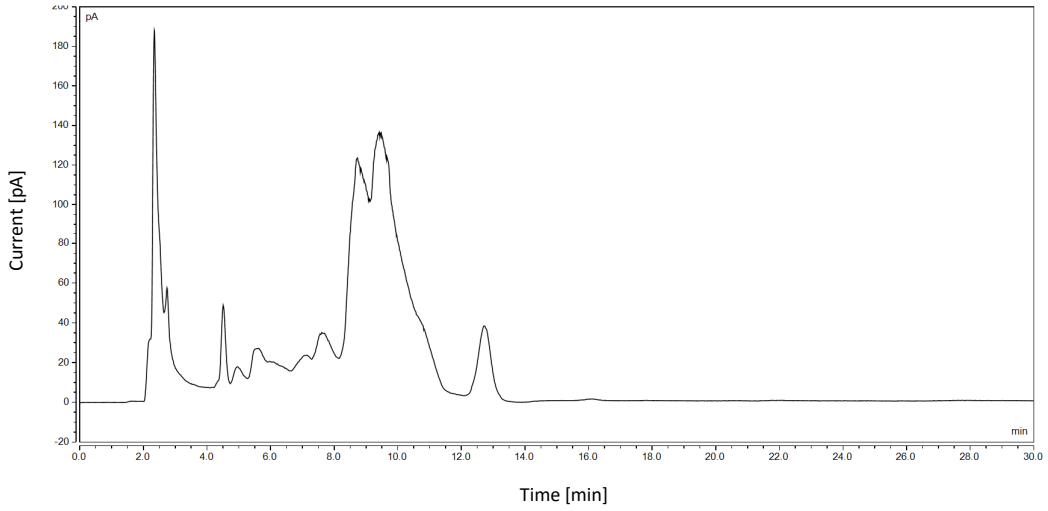

Figure 3.20 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using cold ethanol. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

3.3.3 Plasma protein precipitation using 80% ACN

We used 80% ACN to precipitate plasma proteins. ACN has been proven to be effective in removing plasma proteins as a part of sample preparation for analysis with either liquid chromatography or mass spectrometry. However, due to poor solubility of fucose in ACN, we added water to make solution similar to that of the mobile phase which contain 80% ACN and 20% water. We did simple solubility test by dissolving 5 mg of fucose in 6 mL of 80% ACN and obtained completely soluble fucose solution. Hence, this solution contained approximately 0.8 mg/mL fucose which was considerably higher than the estimated fucose concentration in plasma. High concentration of ACN was expected to precipitate plasma protein while keeping the ability to dissolve fucose.

Plasma protein precipitation using 80% ACN was performed as described under method ***2.2.3.3 Precipitation using 80% ACN***. Figure 3.21 shows chromatogram of the supernatant obtained after precipitation using 80% ACN. Similar to the chromatogram of supernatant obtained after

precipitation using ASSS and ethanol, endogenous compounds were shown to be continuously eluted starting from 2 to around 9 minutes. Hence, both elution and the detection of fucose could potentially be interfered by endogenous compounds from the blank plasma.

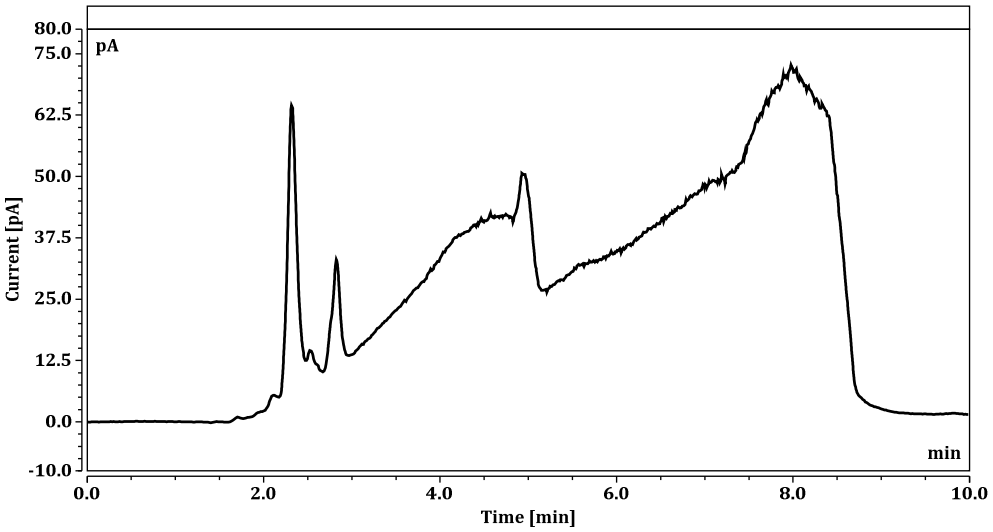

Figure 3.21 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using 80% ACN. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

At this point, we had used three different types of precipitating agents. However, they seemed to be not suitable in improving the separation of blank plasma containing fucose using the method candidate. Hence, the reason for this poor chromatographic separation was probably due the type of chromatographic method used in this study and not the utilised precipitation method. All the precipitating agents used in the current study have been shown to be effective in removing plasma proteins[102-104]. For instance, a study conducted by Rajasekhar et al. suggested that the ACN was effective in precipitating plasma proteins[104]. However, they used ODS (*Octa Decyl Sylane*) column which is a non-polar column instead of a polar column which was used in this study. They also used a mixture of ACN and ammonium acetate buffer solution to elute the sample. Disregarding the type of analyte of interest, different types of columns and

eluents have probably given different response to the ACN-extracted plasma sample, hence different results were obtained. Because of this, we focused on modifying the chromatographic parameters to obtain a suitable method capable of separating fucose from other endogenous compounds present in plasma. Since we preferred to keep using the Asahipak NH2P-50 column in this study, the first attempt was targeted on modifying the mobile phase. This included changing the currently used mobile phase composition, type of elution (whether isocratic or gradient), and finally changed the type of mobile phase and its composition. Precipitation method using 80% ACN was kept, not only because it can precipitate plasma proteins but also was similar to the utilised mobile phase in the method candidate.

We modified the mobile phase to have several different compositions of water and ACN to see the influence of this modification to the elution of fucose-spiked blank plasma. Mobile phase consisted of water and ACN of 30%:70%, 25%:75%, 15%:85%, 10%:90% or 5%:95% were tested. Mobile phase composition was changed through the option available in the instrument controller and was mixed using low pressure gradient mixing mode. We also temporarily used UV detector instead of CAD since there were no significant detection differences between UV and CAD response when a solution containing high concentration of fucose (1 mg/mL) was used. This way we focused on obtaining a method which can appropriately separate fucose and other components present in plasma without necessarily utilising Nitrogen gas as if CAD was used. Solution containing 1 mg/mL of fucose in water was made and 10 µL of this solution was injected to determine retention time of fucose in the tested mobile phase composition prior to analysing fucose-spiked blank plasma. This way, possible interference from the endogenous compounds in plasma on fucose could be determined. Then, further adjustment to mobile phase composition could necessarily be applied until no interfering compounds were observed.

The result of this test showed that mobile phase consisted higher composition of water, with the respective composition of water and ACN (25:75), eluted endogenous compounds from plasma with shorter elution time compared to when mobile phase consisted of Water:ACN (20:80) was utilised. Figures 3.22 and 3.23 show the chromatograms of fucose and plasma supernatant (obtained after precipitation of blank plasma with 80% ACN) respectively. The retention time of fucose was observed to be 5.5 minutes and multiple peaks were eluted (from 2 to 10 minutes) when plasma supernatant was analysed. The multiple peaks were also observed at around fucose retention time when water composition in mobile phase was linearly changed from 25% to 20% in 20 minutes as shown in Figure 3.24.

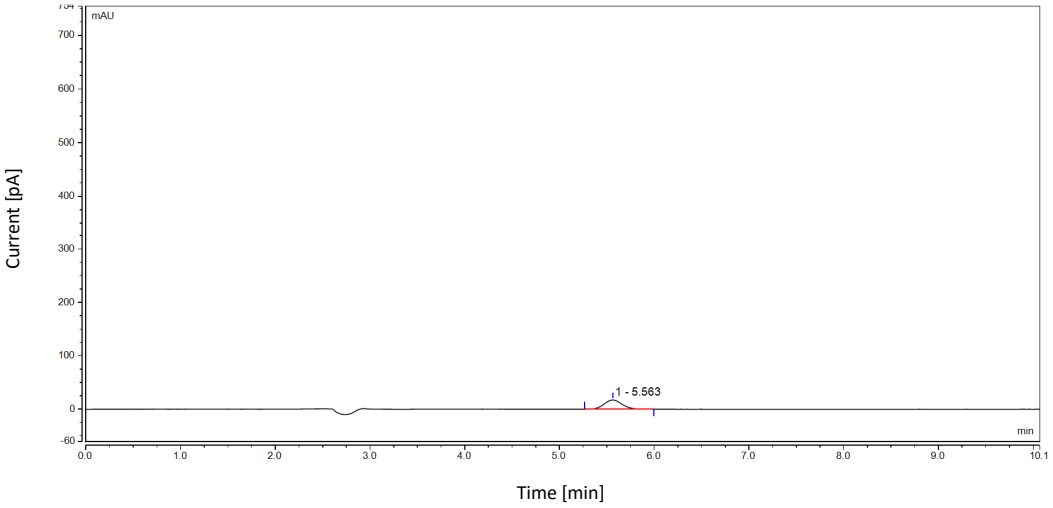

Figure 3.22 Normal phase-HPLC separation of solution containing 1 mg/mL of fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (25:75); detection wavelength 190 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

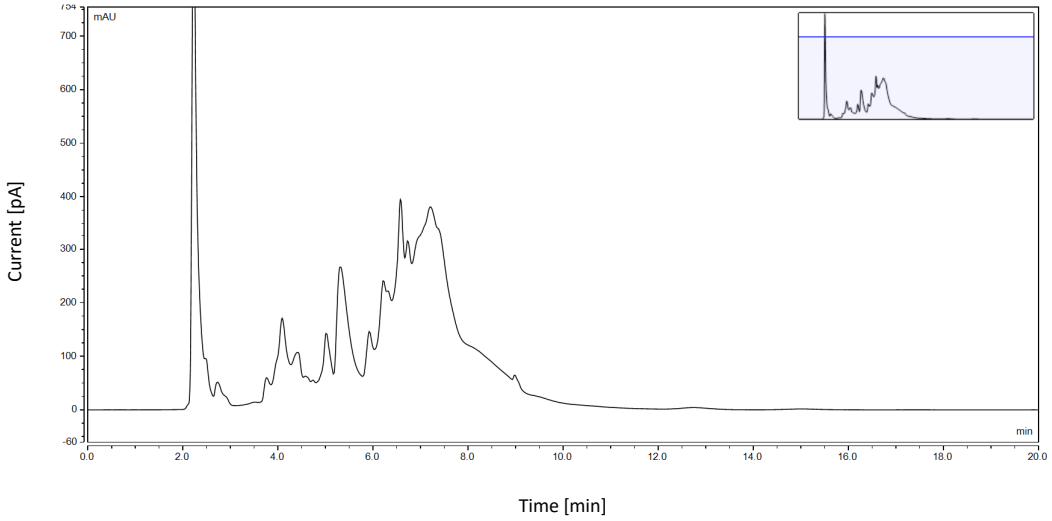

Figure 3.23 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using 80% ACN. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (20:80); detection wavelength 190 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

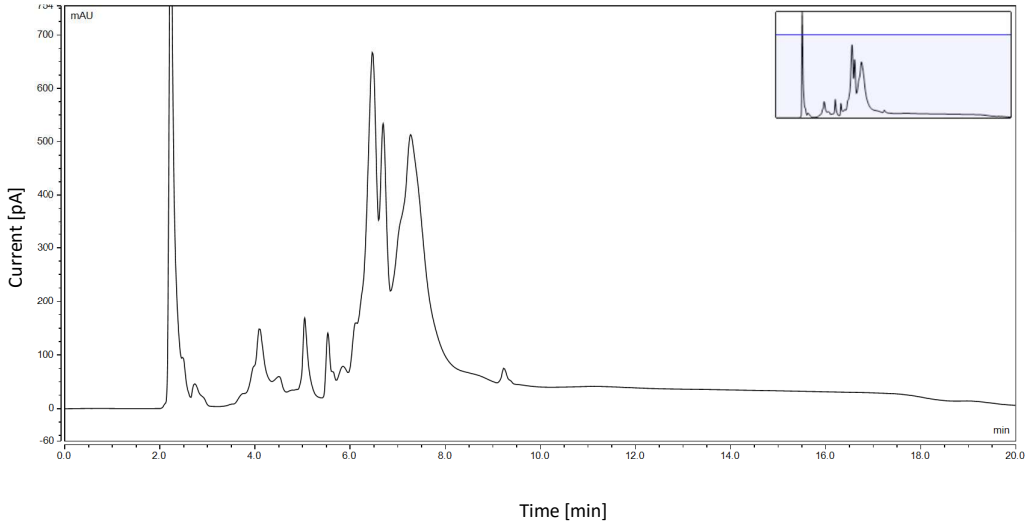

Figure 3.24 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using 80% ACN. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing water and ACN (water was linearly changed from 25% to 20%); detection wavelength 190 nm with diode array detector, flow rate 1 mL/minute, injection volume 10 µL.

On the other hand, decreasing the composition of water in mobile phase to 15% increased elution time of all endogenous compounds. Even longer time was needed to elute these compounds when mobile phase containing 10% or 5% of water was used.

At this point, we concluded that mobile phase consisted of only water and ACN would not be able to resolve fucose and other substances present in plasma from each other with the currently used column. We decided to modify the mobile phase and looked upon the use of salt buffer for this type of column. Mobile phase consisted of water and ACN resolved the injected sample mainly based on polarity. Asahipak NH2P-50 4E is an amino column and its stationary phase composed of polyamine attached to hydrophilic polymer gel. The availability of amine group brings polar property to the column which is one of the reasons it can retain polar compounds such as monosaccharides. Elution of monosaccharides occur in the order of increasing polarity. Hence, compounds with more polarity retains for a longer period of time than those with less polarity. Amine groups also creates an alkaline environment in the column which offers additional advantages in the analysis of monosaccharides with anomeric carbon atom. It prevents the separation of monosaccharide anomers which can cause split peak when analysis is performed at room temperature[105].

On the other hand, salt buffer in mobile phase can change the chemical properties of the column's stationary phase. Hence, the separation of compounds occur not only due to polarity but also because of the interaction between mobile phase, sample and stationary phase[98]. Introduction of mobile phase containing buffer, particularly ammonium acetate, alters the separation characteristic of the column. Amine groups can also be considered as an ion exchange resin, more specifically anion exchange resin. Composition of ammonium acetate in mobile phase and its pH determines the ratio of non-protonated/protonated amine group in the stationary phase. Ammonium acetate solution with high pH gives a greater ratio of non-protonated/protonated amine, uses low elution volume and produces high and sharp peaks when used with Shodex Asahipak column [98,105,106].

The utilisation of ammonium acetate in this study was in line with several considerations. First, ammonium acetate has been commonly used as buffer additives in mobile phase. Second, as mentioned earlier, ammonium acetate can alter the ratio of non-protonated/protonated amine groups present on the stationary phase of Shodex Asahipak column. This would then change the separation characteristic of the column. Third, buffering capacity of ammonium acetate solution can be obtained when the pH is adjusted to 3.8-5.8. Maximum buffering capacity is at pH 4.75. This pH value is considered appropriate when the CAD is employed as a detector. The CAD is compatible when the pH of mobile phase is below 7.5. Four, ammonium acetate easily vaporises which is one of the requirements for buffer additives when CAD is utilised. Therefore, based on these criteria, we decided to use ammonium acetate as buffer additive in mobile phase.

The addition of ammonium acetate buffer to the mobile phase significantly improved separation of fucose and other compounds in the plasma compared to when the mobile phase consisted only water and acetonitrile. Nonetheless, it appeared that acidic environment in the column affected the separation of some of the components presents in plasma but not fucose. There was no major change in the retention time of fucose when ammonium acetate was introduced into mobile phase. In contrast, some compounds were observed to be eluted faster than other.

The option to use salt buffer other than ammonium acetate was limited. There are other buffering agents recommended by the manufacturer of the utilised CAD detector: TFA, formic acid and ammonium formate. However, we preferred to use ammonium acetate among others since its maximum buffering capacity can be obtained at a bit higher pH value; pH 4.76

compared to 0.3 for TFA and 3.75 for formic acid and ammonium formate. Adjusting mobile phase to a very low pH value as a result of the utilisation of TFA, formic acid or ammonium formate could drastically change the initial basic environment of the column. This could probably decrease the lifetime of the column, while the expected figure of separation obtained by the use of these buffer agents would not significantly improved than that of ammonium acetate.

The option to use phosphate buffer was not also possible to be taken. This is because phosphate is non-volatile and hence not compatible with CAD. The solubility of phosphate buffer in ACN is also considerably low. Hence, it would have limited the option to alter the composition of mobile phase or to adjust the strength of phosphate buffer since the mobile phase was also composed of ACN. Therefore, based on these considerations, we decided to use ammonium acetate as buffer additive in mobile phase.

3.4 Optimisation of mobile phase: the utilisation of ammonium acetate

We initially used a 5 mM ammonium acetate solution. The pH of ammonium acetate solution was adjusted to 4.75 with diluted acetic acid. This solution was filtered using a 0.45 µm pore size filter paper and was degassed before use. The mobile phase was composed of 20% of 5 mM ammonium acetate solution (pH 4.75) and 80% of ACN. Other chromatographic parameters were kept the same to the method candidate described in sub chapter 3.2. The column was equilibrated by flushing mobile phase for 30 minutes. We used fucose-spiked blank plasma with quite high concentration of fucose (1 mg/mL) in order to easily observe fucose peak and the quality of separation from other interferences.

Initially, fucose solution (500 µL) was added to the blank plasma (500 µL). The resulting solution was then precipitated using 80% ACN as described above (sub chapter 3.3.3). The supernatant was taken and analysed using HPLC. The chromatogram obtained after injection of plasma containing fucose is shown in Figure 3.25. It can be seen from the chromatogram that the utilisation of ammonium acetate buffer solution in mobile phase significantly improve the separation between fucose and endogenous compounds from blank plasma. Fucose was eluted at around 7.5 minutes. However, it was co-eluted with other unknown compounds present in plasma. The co-eluted fucose peak was confirmed by comparing the retention time of co-eluted peak with the retention time of fucose (Figure 3.26). This showed that the separation of fucose from other compounds was improved with a mobile phase containing ammonium acetate buffer compared to when mobile phase consisted of only water and ACN was utilised. Endogenous compounds which were previously observed to be eluted from 2 to 12 minutes (figure 3.23), this time were eluted faster to until around 7 minutes (ended slightly before fucose which we thought was observed at around 7.5 minutes).

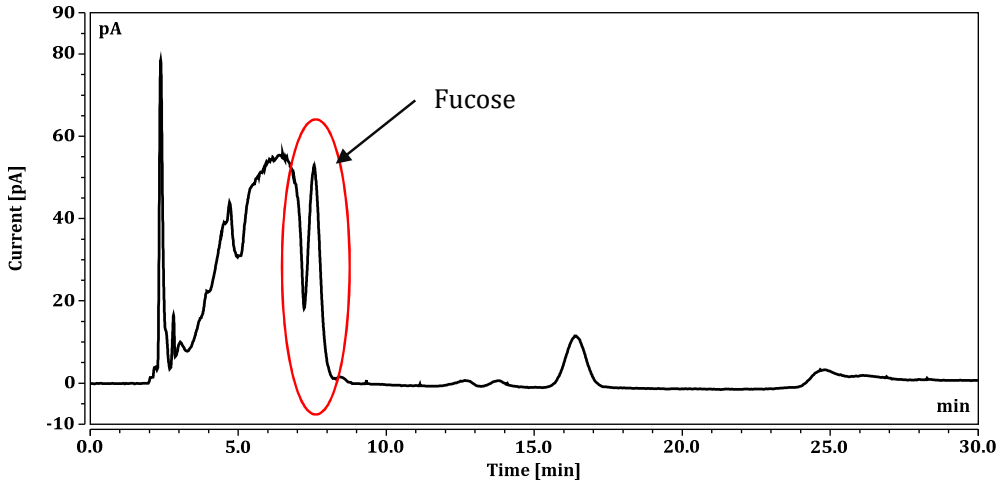

Figure 3.25 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using 80% ACN. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

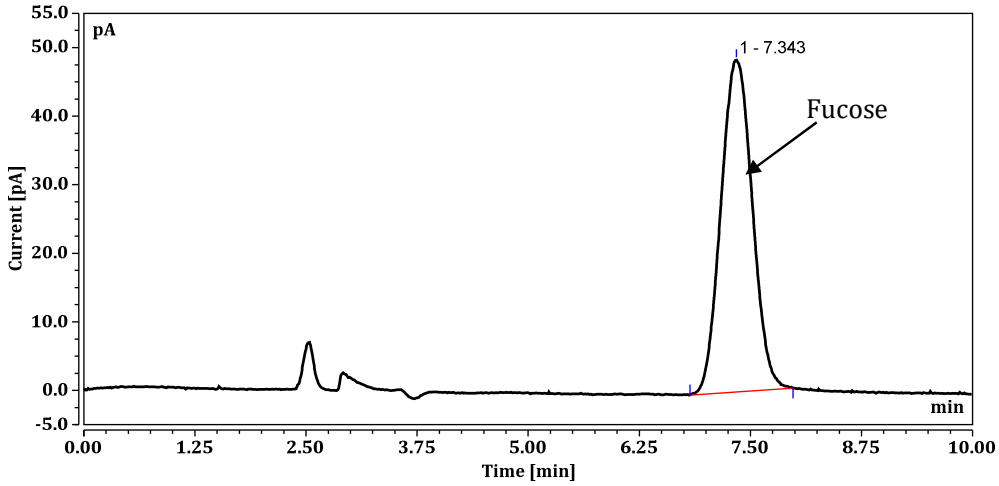

Figure 3.26 Normal phase-HPLC separation of solution containing fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

We again let the mobile phase run through the column for another 1 hour to give more time for the column to be fully conditioned with the mobile phase before reinjecting the same sample. Figure 3.27 shows the chromatogram of the same supernatant sample which was reinjected after another 1 hour of column conditioning. It can be seen from the chromatogram that, compared to figure 3.25, fucose was clearly separated from other components in the sample. The resolution between fucose and the earlier eluted compounds was also significantly increased. This suggested that the utilisation of ammonium acetate in mobile phase has improved separation between fucose and other components present in plasma. Hence, we kept this mobile phase and made further adjustment to its composition and other chromatographic parameters to obtain the most suitable chromatographic system. Several parameters were adjusted which include adjustment of mobile phase composition, the pH of ammonium acetate solution and the strength of ammonium acetate solution. To improve the detection of fucose, two parameters in CAD were also modified, namely power function (PF) and filter value (FV).

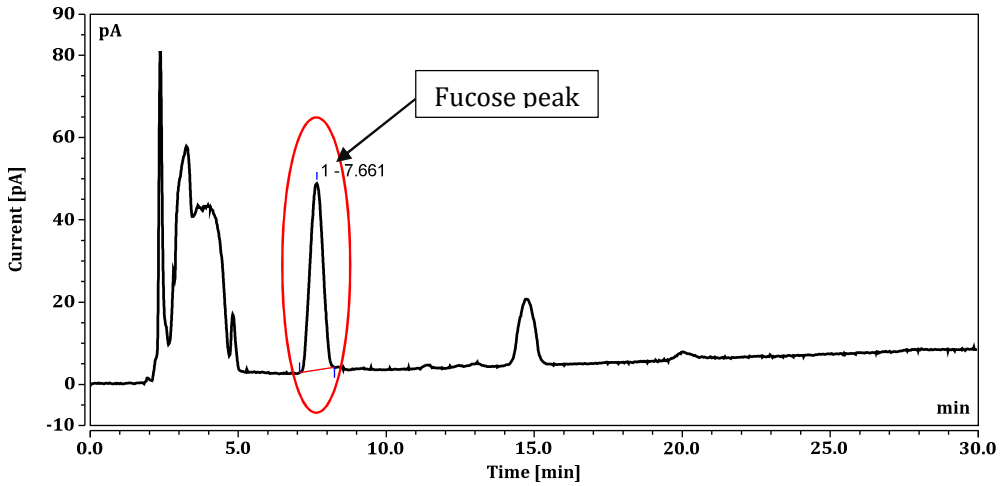

Figure 3.27 Normal phase-HPLC separation of supernatant obtained after precipitation of blank plasma using 80% ACN (re-injection). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

3.4.1 Influence of different mobile phase composition on chromatographic separation

Changes in mobile phase composition and its influence on the separation of fucose and other components in plasma sample was investigated. Mobile phase consisted of 5 mM Ammonium acetate (pH 4.75) and ACN (20:80) respectively, resolved fucose-spiked blank plasma sample without significantly changing the retention time of fucose as to when mobile phase consisted of water and ACN (20:80) was utilised. Increasing buffer composition so that the mobile phase consisted of 5 mM ammonium acetate (pH 4.75) and ACN (25:75) and (30:70) respectively, shortened elution time of fucose and other components and also increased the intensity of fucose peak. Fucose was observed to be eluted at around 6 minutes, approximately 1.5 minutes faster compared to when mobile phase containing ammonium acetate buffer and ACN with the composition of (20:80) was used. However, when blank plasma was subjected to analysis using both of these mobile phase compositions, a peak was observed suggesting a detectable compound but was not fucose despite its close retention time to fucose. Consecutively injected fucose solution and blank plasma confirmed this conclusion (figure 3.28 and 3.29). It can be

seen from both the chromatograms that the retention time of the compound observed in the injected blank plasma was 0.2 minutes shifted compared to the retention time of fucose. Another confirmation attempt was performed by injecting fucose-spiked blank plasma sample. Hence, we thought that with both mobile phase composition, fucose was probably interfered by other component in the plasma. This also suggested that the mobile phase consisted of 5 mM ammonium acetate pH 4.75 and ACN with the composition of (25:75) and (30:70) respectively, could not be used.

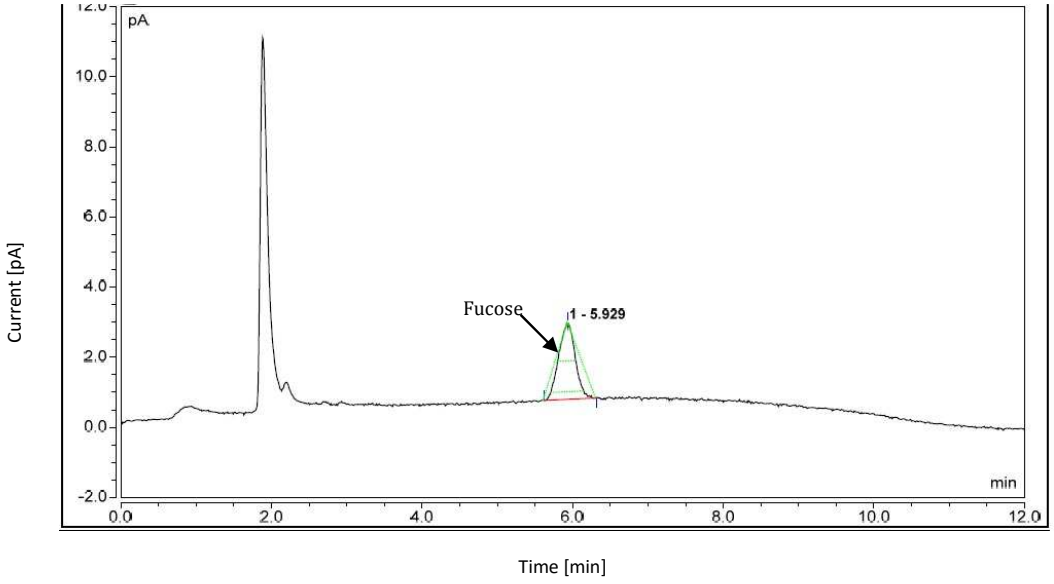

Figure 3.28 Normal phase-HPLC separation of solution containing fucose. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

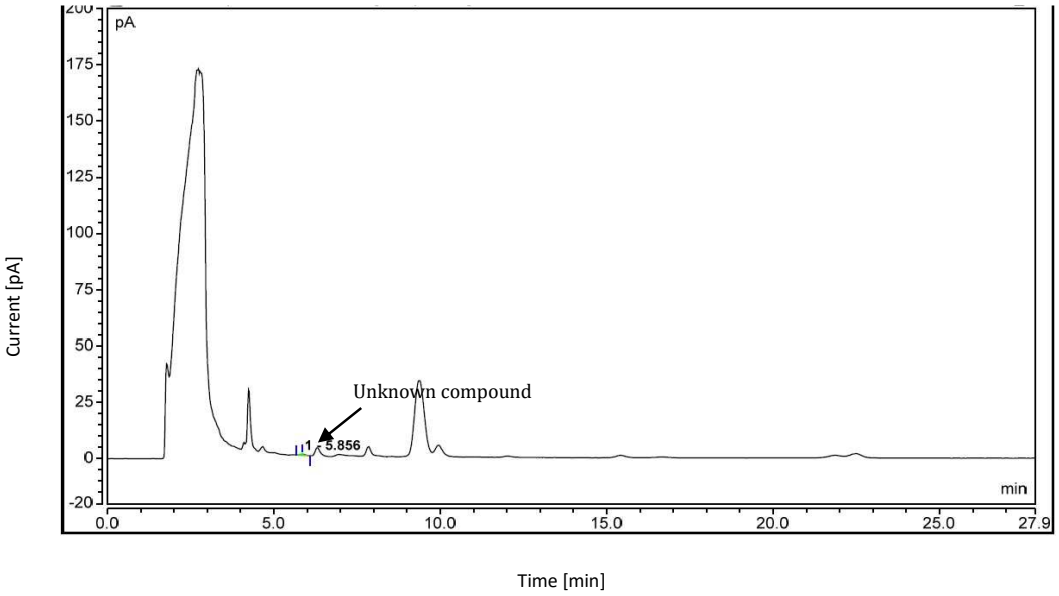

Figure 3.29 Normal phase-HPLC separation of blank plasma. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (25:75); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

In contrast, adjusting mobile phase to contain 5 mM ammonium acetate pH 4.75 and ACN with the composition of (15:85) and (10:90) respectively, increased retention time of fucose. Longer running time was also needed to completely elute the sample. The intensity of fucose peak and other detectable compounds was also decreased. This was similar to that observed when mobile phase consisted of only water and ACN was used as described under sub chapter 3.2. Thus, we decided to use mobile phase consisted of ammonium acetate buffer solution and ACN with the composition of (20:80) which gave optimum separation of fucose and other endogenous compounds.

3.4.2 Influence of different pH value of Ammonium acetate solution on chromatographic separation

It has been proposed that altering the pH of ammonium acetate solution in mobile phase to some extent will influence the ratio of non-protonated/protonated amine group in the column's stationary phase. Therefore, the influence of this pH to the separation of fucose from other

compounds present in plasma was investigated. We limited pH selection of ammonium acetate solution to be in the range of its buffering capacity from 3.8 to 5.8. Three different pH values were chosen: 4.0, 4.75, and 5.0. The chromatograms obtained using a mobile phase consisted of 5 mM Ammonium acetate pH 4.75 and ACN (20:80) was used as reference to determine the influence of pH on fucose separation from other compounds present in plasma supernatant. Higher pH value of ammonium acetate was expected to shorten retention time, increase intensity and sharper fucose peak. On the other hand, increase in retention time, lower intensity and wider fucose peak was expected with lower pH value.

Figure 3.30 shows chromatograms of fucose-spiked blank plasma samples containing 1 mg/mL of fucose. This is an overlay view of 6 chromatograms showing the samples eluted with mobile phase containing ammonium acetate solution with different pH (4.0, 4.75 and 5.0). It is clear from the chromatograms that fucose was eluted at similar retention time in all three tested pH value of ammonium acetate solution. The intensity of fucose peak was the highest when the pH value of ammonium acetate was adjusted to 4.75 and 5.0. However, this was not significantly different to when pH value of 4.0 was chosen, though the observed height of fucose peak was a bit shorter. Thus, it can be concluded that changing pH value of ammonium acetate solution by 0.5 did not significantly change fucose retention time and peak intensity or height. The reason for this, we thought, probably because of the narrow gap between the tested pH values. The range of the tested pH values was not wide enough to produce significantly different ratio of non-protonated/protonated amine group in the column. We, therefore, decided to use ammonium acetate solution pH 4.75, the pH value at which its buffering capacity is the highest.

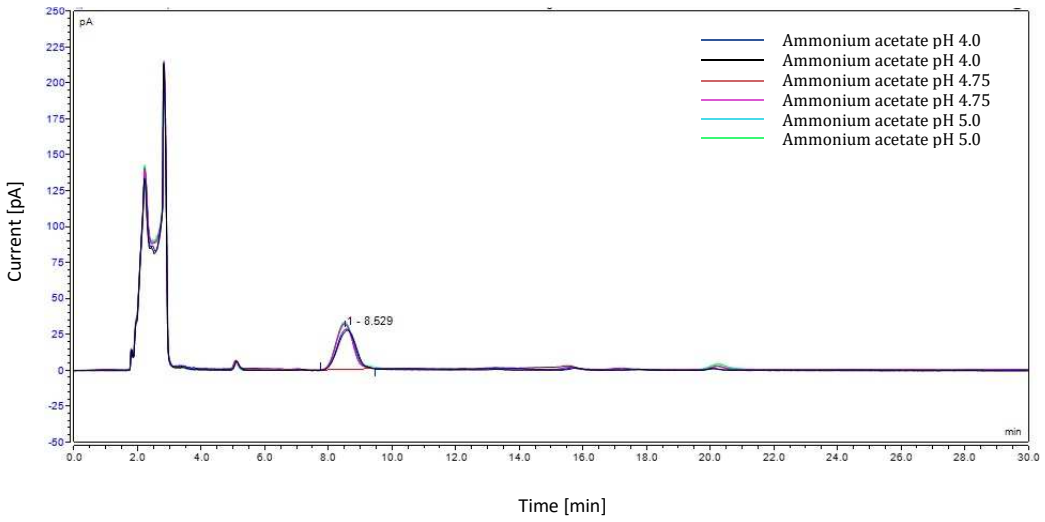

Figure 3.30 Normal phase-HPLC separation of fucose-spiked blank plasma (overlay view of six chromatograms). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate with three different pH value (4.0, 4.75 and 5.0) and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

3.4.3 Influence of different strength of Ammonium acetate solution on chromatographic separation

Ammonium acetate solutions with different concentration (2.5 mM, 5 mM, 10 mM and 25 mM) were used to determine the influence of its strength on the separation of fucose in fucose-spiked blank plasma samples. The pH of ammonium acetate solution was adjusted to 4.75. Mobile phase consisted of each different strength of ammonium acetate solution and ACN (20:80) was used to elute fucose-spiked blank plasma sample. Mobile phase was allowed to run through the column for 1 hour to fully equilibrate the column. We avoided using high concentration of ammonium acetate due to its low solubility in ACN which can probably cause precipitation in the column and, hence, disturb the sample elution. It was expected that as the strength of the buffer changed, change to the separation of fucose and other compounds in the sample would also occurred. Higher concentration of ammonium acetate in mobile phase can increase the retention of fucose and other components in the sample due to the increase of interaction between the compounds and ammonium acetate occupying column's stationary

phase. However, we observed no change either in fucose retention time or its separation from other components in the plasma sample in the utilised different strength of Ammonium acetate solution. Therefore, we decided to use the lowest concentration, 2.5 mM ammonium acetate solution. This will lower the chance of immiscibility between the buffer solutions with ACN due to its low concentration.

Up to this stage we concluded that mobile phase consisted of 2.5 mM ammonium acetate pH 4.75 and ACN (20:80) was appropriate to be utilised in resolving fucose-spiked blank plasma.

3.4.4 Influence of altering Power Function and Filter value in CAD in improving linearity and resolution

There are two parameters in CAD which can be adjusted to improve the appearance of a chromatogram in an analysis using CAD, namely: Power Function (PF) and Filter Value (FV). Power Function relates to manipulation attempt performed by the system of the instrument to produce better output using chromatographic data. However, this does not mean components separation profile in a sample is also changed[107].

A study has proven that several chromatographic parameters can be changed with the adjusted PF value[107]. Linearity is one such parameters which can be altered by changing PF value. Linearity, at a certain range of analysis, can usually be improved in a higher PF value. Similarly, higher PF value will also increase resolution between compounds. Increasing PF value will narrow an analyte peak-width which will then widen gap between compounds and hence improves resolution. Another change in a chromatogram appearance which can be observed due to this alteration in PF value is the signal to noise ratio. Relates to narrower peak

width and improvement in analytes resolution in a sample when higher PF value is used, this will also increase signal to noise ratio. Analytes peaks will appear to be sharper. This was similar to the result obtained in our study. While the detection correlation between the concentration of the sample and the area shown by the detector was suitably calculated in exponential manner at PF value of 1.0, this was no more valid with higher PF value of 1.5 and 2.0. Lower correlation coefficient was obtained if exponential relation was kept. This created difficulties in choosing appropriate equation showing correlation between area and concentration of the sample if the particular PF value was selected. Whilst considerably good R^2 was obtained in exponential manner when PF value was set to 1.0, neither linear nor exponential equation showed similar correlation coefficient at PF of 1.5 or 2.0.

An equation which suitably correlate area in the chromatogram to the concentration of analyte is important to have perfect quantification of an analyte. Moreover, altering PF value also changed the area of the analyte. The system adjusted the area proportional to the selected PF. It was observed that higher PF gave lower area. It came to the result that at PF 2.0, the injected standard containing 10 ng of fucose gave area of 0.000. However, it is not necessarily be meant that the peak was not detected. In fact, fucose peak was still observed. Nevertheless, the area was not appropriately quantified due to limitation of minimum detected area can be shown by the instrument. Based on these two reasons, we decided to keep PF value at 1.0 with used exponential equation to quantify fucose based on its detected area.

3.5 Digestion of fucoidan

The method which can separate fucose from other endogenous substances present in plasma used Shodex Asahipak NH2P-50 4E column and mobile phase consisted of 2.5 mM Ammonium acetate pH 4.75 and ACN (20:80). The column compartment temperature was 30

°C and the autosampler compartment was 10 °C. Detection was performed using a CAD with PF and FV values set at 1.0 and 3.0, respectively. This method was intended to indirectly quantify fucoidan present in patients' plasma. This was aimed to determine fucoidan concentration in plasma sample by quantifying the amount of fucose present in plasma samples taken before and after oral administration of fucoidan (187.5 mg four times daily) for 3 weeks. To do this, fucoidan must be degraded into its simplest constituting monosaccharides, particularly fucose. Hence, a digestion method was required to completely break down fucoidan into fucose.

At this stage, the utilisation of 80% ACN was expected to completely precipitate plasma proteins. However, the recovery of fucoidan from plasma was yet to be evaluated. The digestion methods were tested with the consideration that the fucoidan could be completely recovered from plasma using 80% ACN. This implied that the most fucoidan would have been present in the supernatant. Therefore, fucose obtained after the tested digestion would be considered only obtained from the digested fucoidan. This is because no endogenous fucose was observed in the supernatant following precipitation using 80% ACN.

Digestion method using a 2 M TFA or 20% TFA was investigated. The performance of each method in digesting fucoidan into fucose was determined by comparing the amount of fucose obtained. A method yielded the most fucose was chosen. It has been reported that fucoidan can also be depolymerised using particular types of enzymes (e.g. fucoidanase). However, we preferred to use the chemical rather than enzymatic degradation because the chemical methods for the degradation of fucoidan have been well established and documented. Different types of fucoidanase enzymes are required for complete digestion of fucoidan. Also, such enzymes are not commercially available. These enzymes are reported to be produced by several types of

Gram Positive microorganisms. Therefore, we were required to first culture the different types of micro-organisms before even extracting the fucoidan degrading enzymes. The extracted enzymes then need to be purified as well as identified before employing them for fucoidan degradation. The candidate was expected to spend more than 12 months to accomplish the above mentioned steps. One of the aims of the study was the complete digestion of fucoidan. This could be achieved with the use of well-established chemical digestion procedures.

3.5.1 Digestion of fucoidan using 0.2 M TFA

Fucoidan was digested using 0.2 M TFA as described in 2.2.5.1. The amount of weighted fucoidan is shown in table 3.5.

Table 3.5 The amount of weighted fucoidan for the determination percentage of fucose present in fucoidan after digestion of fucoidan using 0.2 M TFA

Sample Name	Weighted (Sample + container) (g)	Container weight (g)	Net weight (g)
Fucoidan 1	0.1035	0.0865	0.0170
Fucoidan 2	0.1041	0.0868	0.0173

The amount of fucose yielded after digestion was calculated using a standard calibration curve obtained from the injected fucose standard solutions containing 5, 10, 25, 50 and 100 µg/mL of fucose (Figure 3.31). It was a quadratic equation with the formula of $y = -9.718x^2 + 375.22x - 8.7755$, where y is the amount of the injected fucose in nanogram and x is the related fucose peak area. Correlation coefficient (R^2) was 0.9999.

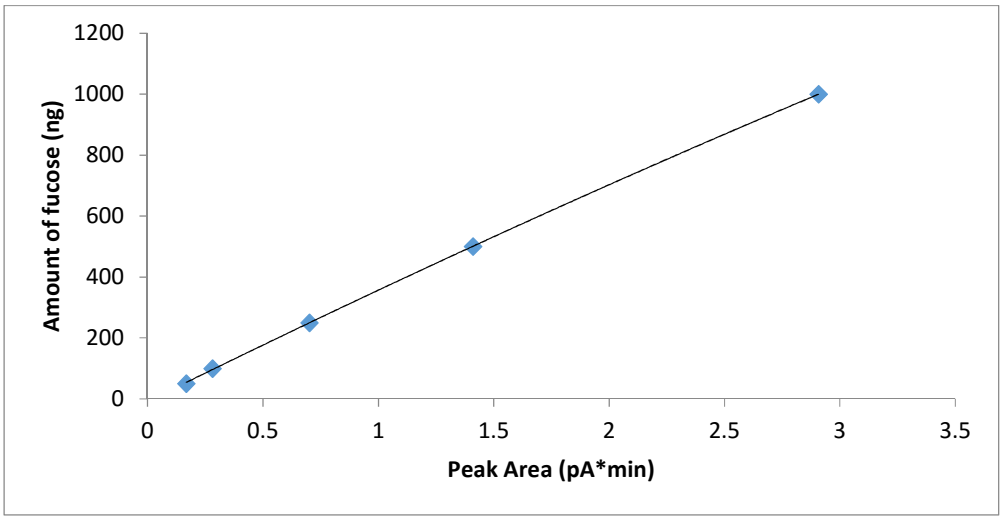

Figure 3.31 Standard calibration curve (peak area vs amount of fucose) using five injected fucose solution containing 5, 10, 25, 50 and 100 µg/mL of fucose

The amount of fucose calculated from the two samples (injected in duplicate) are shown in table 3.6. The concentration of fucose in fucoidan was calculated by dividing the total amount of fucose by the initial amount of fucoidan and was shown in percentage values.

Table 3.6 Percentage of fucose in fucoidan after digestion using 0.2 M TFA

Sample Name	Peak Area	Amount of fucose (ng)	Total amount of fucose (mg)	Fucose percentage in fucoidan (%)
Fucoidan 1a	1.1969	443.95	3.55	20.89
Fucoidan1b	1.1932	442.65	3.54	20.83
Fucoidan 2a	1.1025	410.64	3.29	18.99
Fucoidan 2b	1.0422	389.27	3.11	18.00

3.5.2 Digestion of fucoidan using 20% TFA

Fucoidan was digested using 20% TFA as described in 2.2.5.2. The amount of weighted fucoidan is shown in Table 3.7. These were three fucoidan samples weighted separately and each sample was analysed in single injection.

Table 3.7 The amount of weighted fucoidan for the determination percentage of fucose present in in fucoidan after digestion of fucoidan using 20% TFA

Sample Name	Weighted (Sample + container) (g)	Container weight (g)	Net weight (g)
Fucoidan 1	0.0847	0.0670	0.0177
Fucoidan 2	0.0845	0.0672	0.0173
Fucoidan 3	0.0848	0.0675	0.0173

The amount of fucose obtained after digestion of fucoidan using 20% TFA was calculated using the same standard calibration curve used to determine amount of fucose after digestion of fucoidan using 0.2 M TFA. The percentage of fucose obtained is shown in table 3.8.

Table 3.8 Percentage of fucose in fucoidan after digestion using 20% TFA

Sample Name	Peak Area	Amount of fucose (ng)	Total amount of fucose (mg)	Fucose percentage in fucoidan (%)
Fucoidan 1	1.1523	428.24	3.43	19.38
Fucoidan 2	1.2059	447.12	3.58	20.63
Fucoidan 3	1.2322	456.37	3.65	21.05

Based on the data shown in table 3.6 and 3.8, it can be calculated that the amount of fucose obtained after digestion of fucoidan using 0.2 M TFA was 19.68% ± 1.42% (n=4), while that obtained after digestion using 20% TFA was 20.35% ± 0.87% (n=3). It can be concluded that, though it was not significantly different, the amount of fucose obtained after digestion of fucoidan using 20% TFA was a bit higher compared to that of digestion using 0.2 M TFA. Hence, 20% TFA was then used for the digestion of fucoidan. This value was also consistent to the values reported by Marinova, the manufacturer of fucoidan used in this study.

3.6 Recovery of fucose from fucoidan-spiked plasma in conjunction with precipitation method

Up to this point, we had chosen 80% ACN as the precipitating agent to precipitate plasma proteins. It has been tested that 80% ACN performed well in precipitating plasma protein as described in 3.3.3. Plasma proteins present in 1 mL of blank plasma could be precipitated with

4 mL of 80% ACN. Fucose and other endogenous substances present in supernatant obtained following precipitation of fucose-spiked blank plasma was resolved well with the chromatographic method using a mobile phase consisted of 2.5 mM ammonium acetate (pH 4.75) and ACN (20:80). The utilisation of 80% ACN should also yield high recovery of fucose from fucoidan. This because the expected analyte present in the plasma sample was fucoidan which, probably, has not been degraded into fucose. Therefore, the determination of the recovery of fucose from fucoidan in plasma, after precipitation of plasma protein using 80% ACN was performed. Moreover, fucoidan was also tested to be less soluble in 80% ACN compared to that in MilliQ water. This was proven by a simple test: whilst 5 mg of fucoidan was completely dissolved in 5 mL of MilliQ water, precipitation was observed when it was dissolved in the same volume of 80% ACN. This would likely decrease the chance for fucoidan to be completely recovered from plasma using 80% ACN.

The recovery of fucose was investigated after precipitation of fucoidan-spiked blank plasma has been performed. Fucoidan solution (1 mL) containing 10 mg of fucoidan was added into blank plasma (1 mL) or Milli-Q water (1 mL). Precipitation was then performed as described in 3.3.3. Fucoidan solution (1 mL) in Milli-Q water was used as control. Digestion using 20% TFA was also performed to the dry samples obtained from the supernatant of both the sample and control as described in 2.2.5.2. The final solution was then subjected to HPLC analysis. The results are shown in Table 3.9.

Table 3.9 Recovery of fucose from fucoidan-spiked blank plasma after precipitation using 80% ACN

Sample name	Fucose peak area	Percentage recovery of fucoidan (%)
Control 1	0.0090	Considered as 100%
Control 2	0.0096	
Sample 1	0.0001	1.08
Sample 2	0.0007	7.53

Based on the data shown in table 3.9, it can be calculated that the average peak area from the control samples was 0.0093 and the average area for the samples was 0.0004. This data shows that, as per the detected fucose peak area, recovery of fucose from fucoidan-spiked blank plasma after the performed precipitation using 80% ACN was very low (average of 4.30%, n=2). This was probably caused by the low solubility of fucose and fucoidan in 80% ACN. The addition of this precipitating agent probably caused fucoidan to precipitate along with plasma proteins. Hence, the small amount was recovered in the supernatant. This also suggested that fucoidan was precipitated, limiting the use of supernatant as the final sample due to low recovery of fucoidan. Another option was to use other precipitating agents such as ethanol and acetone. However, fucoidan in ethanol or acetone was found to be insoluble as well.

To overcome the above mentioned problems, we decided to analyse the precipitates obtained after precipitation of water or fucoidan-spiked blank plasma. Referred to the results obtained following precipitation using 80% ACN, fucoidan was expected to be mostly precipitated. However, since 80% ACN also contain around 20% of water which created chance for fucoidan to dissolve and went to supernatant phase, we decided to use pure ACN to precipitate plasma proteins as well fucoidan.

3.6.1 Recovery of fucose from fucoidan-spiked blank plasma after precipitation using ACN

In this test, fucoidan solution (200 µL) containing 1 mg/mL of fucoidan was added into a 15 mL centrifuge tube containing blank plasma (500 µL). Precipitation using ACN was performed as described in 2.2.3.4. The precipitate was then subjected to digestion using 20% TFA. We tried two different approaches in the way we use 20% TFA to digest protein precipitate and precipitated fucoidan (method 1 and method 2). In method 1, the digested

solution was evaporated to dryness and then Milli-Q water (1 mL) was added to the dry sample. In method 2, MilliQ water (3 mL) was added to the solution, vortex mixed and centrifugation at 3500 rpm for 20 minutes. The supernatant was carefully taken and then evaporated to dryness. Milli-Q water (1 mL) was then added to the dry sample. Final solution from method 1 and method 2 was subjected to HPLC analysis to determine the recovery of fucose from fucoidan-spiked blank plasma.

Table 3.10 shows the percentage of fucose recovered. The Recovery of fucose was calculated using the following equation.

$$\frac{(m_s - m_b)}{m_c} \; x \; 100\%$$

Where m_s was the amount of fucose from the sample, m_b was the amount of endogenous fucose from blank plasma and m_c was the amount of fucose from control. This also suggested, that the recovery of fucose was calculated based on the detected fucose area. The amount of fucose was calculated using the standard calibration curve as described in 3.5.1.

Table 3.10 Recovery of fucose from fucoidan-spiked blank plasma after precipitation using ACN

Sample Name	Peak Area	Amount of fucose (ng)	Total amount of fucose (µg)	Percentage recovery of fucoidan (%)
Sample 1a	1.7464	634.42	63.44	93.21
Sample 1b	1.7185	624.88	62.49	91.10
Sample 2a	1.6961	617.22	61.72	89.41
Sample 2b	1.6690	607.94	60.79	87.35
Control 1	1.2059	447.12	44.71	Considered as 100%
Control 2	1.2322	456.37	45.64	
Blank	0.5531	213.34	21.33	

It can be seen from table 3.10 that the recovery of fucose from fucoidan-spiked blank plasma in method 1 was 92.16% ± 1.49% (n=2) and method 2 was 88.38% ± 1.45% (n=2). The results

indicated that the approach taken to analyse precipitates rather than supernatant gave much higher recovery (92.16%) compared to that of analysing the supernatant after precipitation with 80% ACN (recovery of 4.30%). This also confirmed the suitability of ACN as the precipitating agent to precipitate both plasma protein and fucoidan. Furthermore, based on the result shown in table 3.10, method 1 gave higher recovery compared to that of method 2. Hence, this method was then validated according the guidelines provided by the International Conference on Harmonisation before applying it to analyse patients' samples taken before and after oral administration of fucoidan.

3.7 Method Validation

The linearity estimated by correlation coefficient (r^2) of a calibration curve for fucose peak (n=6) over a range of 5 to 100 µg/mL was greater than 0.994 over a period of 5 days. The assay performance was determined using a fucose peak. The intra- and inter-day precision relative standard deviations (RSDs) for fucose peak were less than 5.0% (n=6) and 3.5% (n=5), respectively. The intra- and inter-day accuracy RSDs were less than 4.6 and 4.0% respectively. The Intra- and inter-day retention time RSDs for fucose peak were less than 2.1% (n=6) and 1.9% (n=5), respectively. The peak area (RSD) of fucose (n=5) investigated after slightly altering three of the chromatographic parameters (the strength of ammonium acetate buffer, pH value and mobile phase composition) was less than 5.0%.

Limit of detection (LOD) and limit of quantitation (LOQ) of the method was investigated using the mean values of three calibration curves. The calculation was performed using the following formula for LOD = (3.3 σ)/S and for LOQ= (10 σ)/S; where σ is the mean standard deviations of the y-intercept and S is the slope of the curve. Limit of detection was calculated to be 2.28 ng and limit of quantitation was 6.92 ng. The reason for using high fucose concentrations was

due to the existence of endogenous fucose in patients as well as blank plasma samples. Also, based on the polarity and size of fucoidan, it was predicted that the amount of endogenous fucose would be greater than the amount of fucose absorbed after oral administration of fucoidan. Hence, the fucose standards were prepared in a way that that the fucose coming from the absorbed fucoidan can still be detected despite the existence of the endogenous fucose.

Table 3.11 Summary of the result of the performed method validation

The tested validation parameters		**Results**	
Linearity		(r^2) > 0.994	Tested using calibration curve of fucose over a range of 5 to 100 µg/mL over a period of 5 days
Precision			
	Intra- day	RSDs < 5.0%	n=6
	Inter-day	RSDs < 3.5%	n=5
Accuracy			
	Intra-day	RSDs < 4.6%	
	Inter-day	RSDs < 4.0%	
Retention time			
	Intra-day	RSDs < 2.1%	n=6
	Inter-day	RSDs < 1.9%	n=5
Peak area		RSDs < 5.0%	n=5

The plasma supernatant containing fucose (n=5) was stored at -20 °C and the concentration of fucose was determined after 0, 7, 14, 21 and 31 days. The peak area of fucose in a sample stored at -20 °C for 0, 7, 14, 21 or 31 days was found to be 20.12, 20.98, 19.77, 21.06 or 20.66. These results indicated that fucose did not undergo degradation when stored at -20 °C for a period of 31 days.

The chromatogram obtained from the analysis of water (blank), fucose standard solution in water (control), blank plasma and patient's plasma sample is shown in figure 3.32, 3.33, 3.34 and 3.35, respectively.

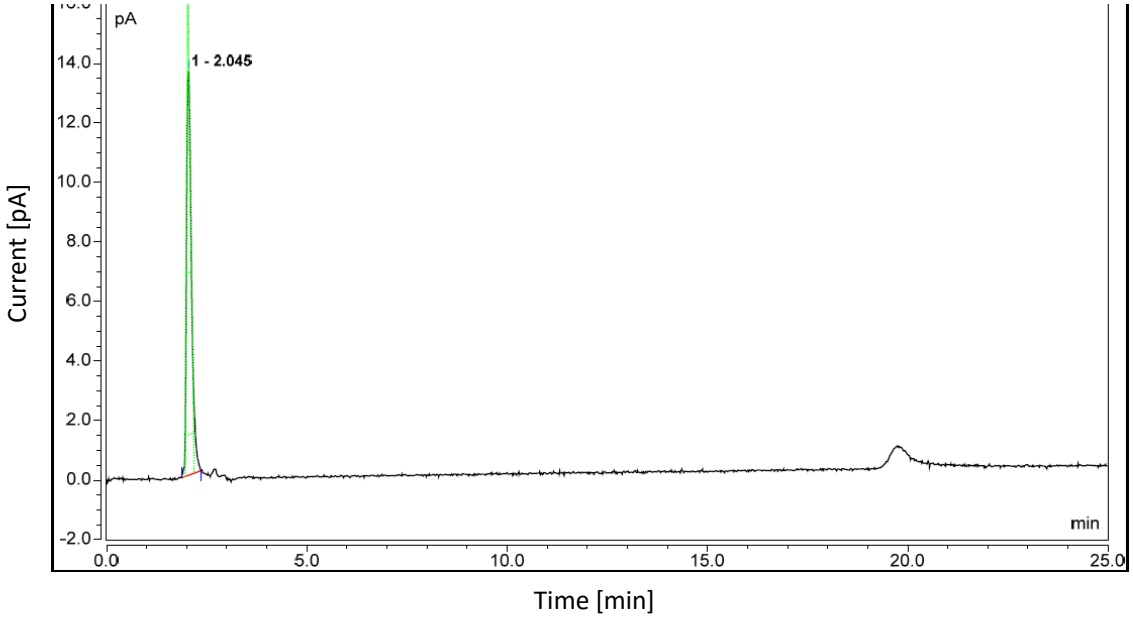

Figure 3.32 Normal phase-HPLC separation of water. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

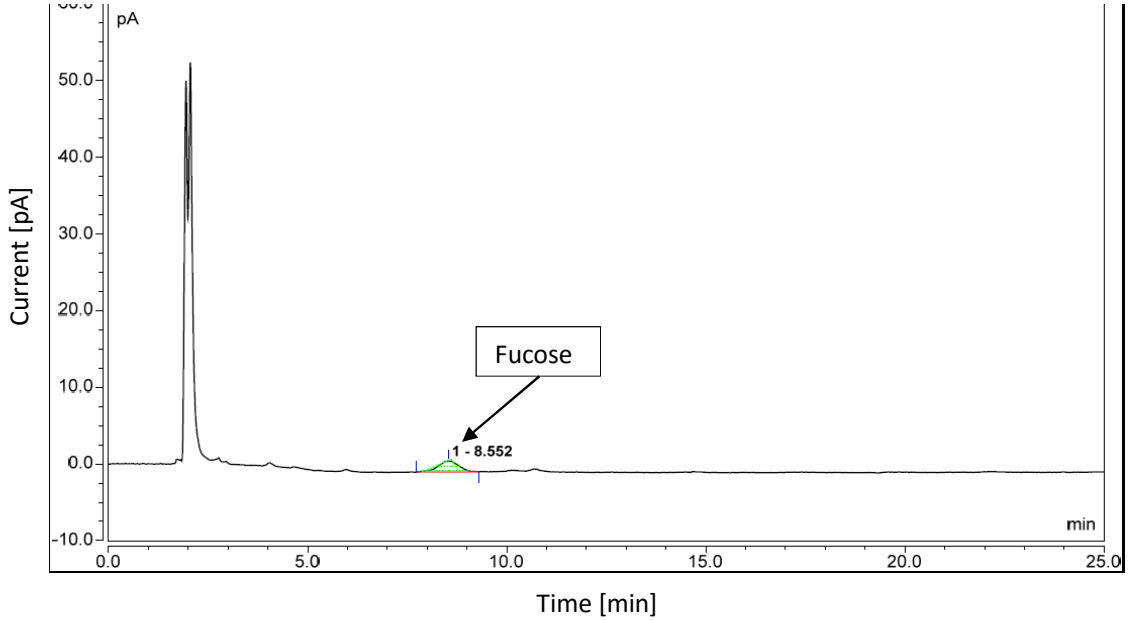

Figure 3.33 Normal phase-HPLC separation of fucose standard solution (control). Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

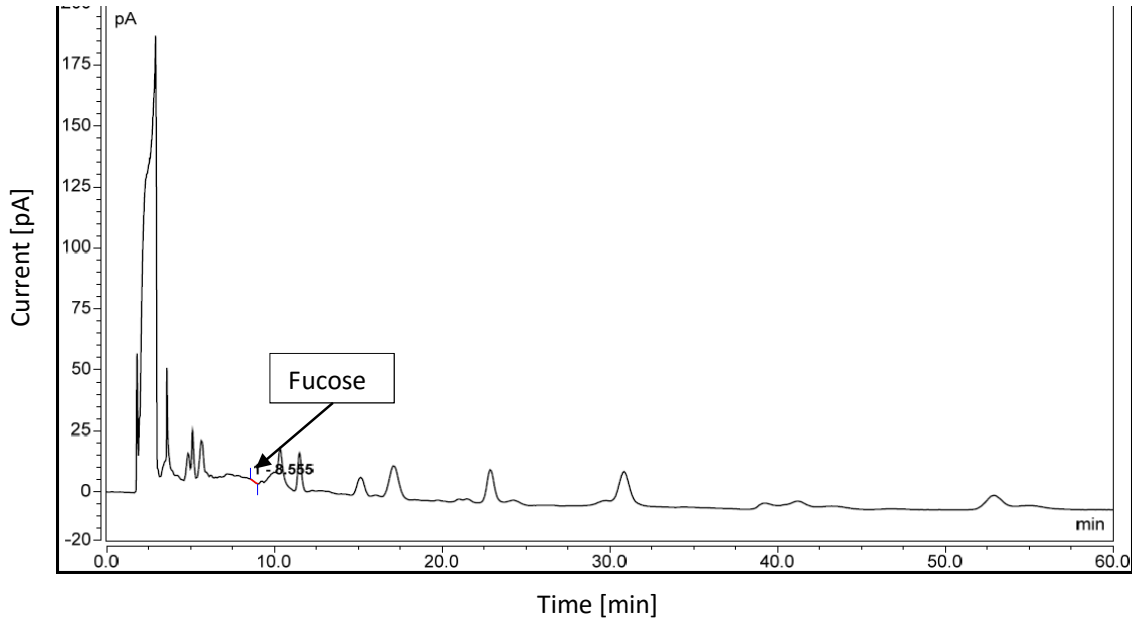

Figure 3.34 Normal phase-HPLC separation of blank plasma. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

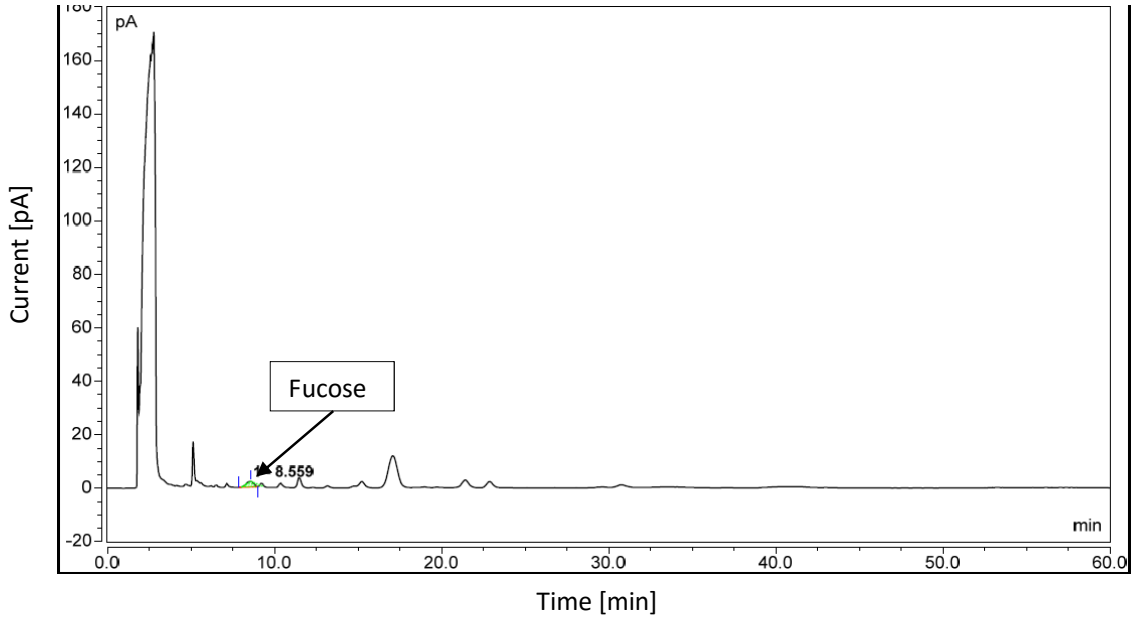

Figure 3.35 Normal phase-HPLC separation of patient's plasma sample. Experimental conditions: Shodex Asahipak NH2P-50 4E (250_4.6 mm), 5 µm; eluent containing 5 mM Ammonium acetate pH 4.75 and ACN (20:80); detection with CAD, flow rate 1 mL/minute, injection volume 10 µL.

3.8 Patients' samples analysis

The developed method was utilised to determine the concentration of fucose in plasma samples obtained from healthy volunteers and from patients with breast cancer (pre- and post-administration of oral fucoidan). The patients' plasma samples were stored under -20°C, four weeks before the analysis was performed. All plasma samples were precipitated using ACN as described in sub chapter 2.2.3.4. The precipitates were then treated with 20% TFA to degrade fucoidan into fucose. The samples were then subjected to HPLC analysis using the developed method. Subsequently, the concentration of fucose was then calculated based on the detected peak area.

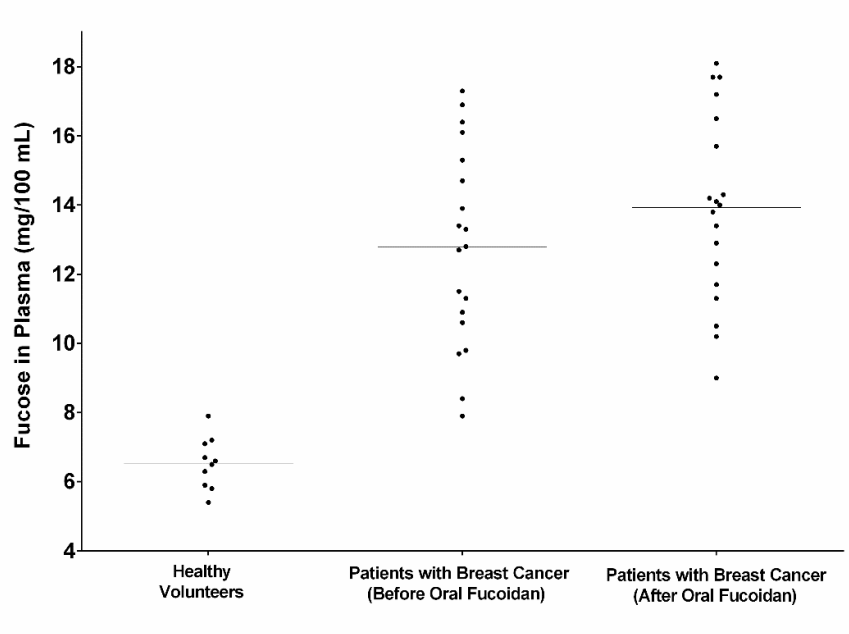

Figure 3.36 Distribution diagram of fucose concentration in plasma sample from healthy volunteers and patients with breast cancer (before and after administration of fucoidan)

As shown in Figure 3.36, the fucose concentration ranged from 5.4 to 7.9 mg/100 mL in healthy volunteers with the mean value of (6.5 ± 0.74) mg/100 mL (n=10). The detected fucose was considered as endogenous fucose since healthy volunteers were not treated with oral fucoidan. This data was similar to a reported result in a study suggesting fucose level in a normal health control to be in the range of 3.42 to 9.05 mg/100 mL with an average value of 6.05 mg/100 mL [108]. Another study has reported the fucose levels in healthy subjects to be in the range of 4.3 to 7.1 with an average value of 5.3 mg/100 mL[109].

Fucose levels were observed to be higher in plasma samples obtained from patients with breast cancer either pre- or post-administration of fucoidan compared to that of normal healthy volunteers. Fucose concentration ranged from 7.9 to 17.3 mg/100 mL with an average value of (12.8 ± 2.86) mg/100 mL in the patients' plasma sample before the administration of fucoidan. Elevated plasma fucose levels in patients with particular pathological states such as breast carcinoma and other pathological states have been reported earlier[108,110-119]. It was also concluded that the rise in fucose concentration was free from particular type of illnesses since it was also found to occur in patients with tuberculosis and liver cirrhosis[109]. Therefore, the comparably high fucose levels in the plasma samples obtained from patients with breast cancer utilised in this study could be justified due to pathological condition of the patients.

Figure 3.36 also shows the concentration of fucose in patients before and after administration of oral fucoidan. The average fucose concentration in all patients' plasma samples was observed to be (13.9 ± 2.71) mg/100 mL or increased by 1.1 mg/100 mL (n=19) after oral fucoidan was administered.

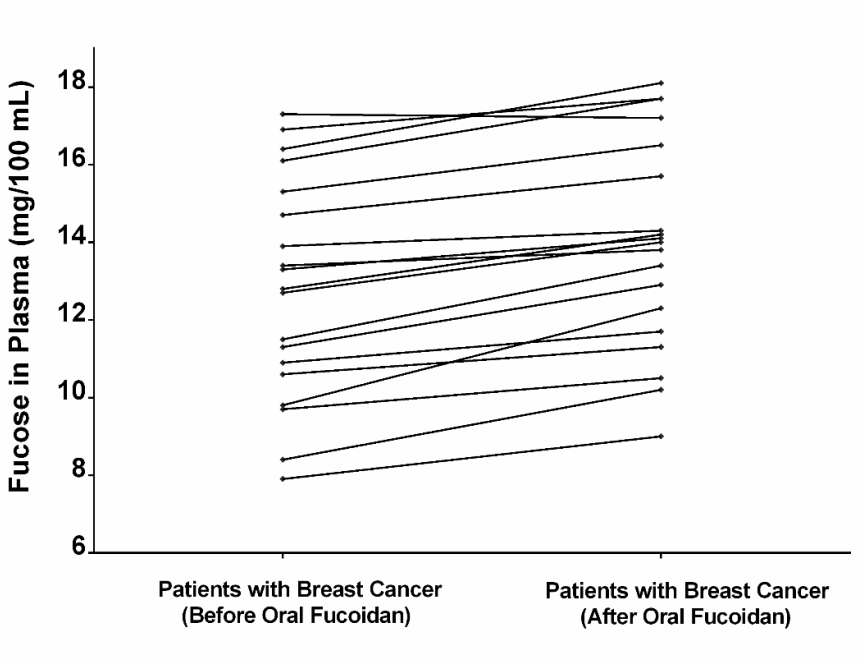

Figure 3.37 The amount of detected fucose in mg/100 mL in each plasma samples from patients with breast cancer before and after administration of fucoidan

Figure 3.37 shows the concentration of fucose before and after the administration of fucoidan in each patients' plasma samples. It can be clearly seen that fucose concentration elevated in all but one patients plasma samples. The absorption of fucoidan and endogenous fucose metabolism in each patient was different. This can be observed by the different initial endogenous plasma fucose concentration between one patient to another and also the different values of elevated fucose concentration after oral fucoidan was administered. This also was probably the cause of the slightly (0.1 mg/100 mL) decreasing plasma fucose level shown in one patient after the administration of oral fucoidan. Nonetheless, the overall result implies the consistency of the data obtained in this study. Therefore, it can be suggested that the administration of fucoidan has caused an increase in fucose concentration. This can also be interpreted that fucoidan was absorbed following oral administration.

The amount of the absorbed fucoidan was then calculated using the available fucose concentration from each patient's plasma samples. Considering the initial endogenous fucose detected in plasma samples from patients with breast cancers differed to that of normal healthy volunteers; fucose level in the patients was then considered as the initial fucose level. This fucose concentration was used to subtract fucose concentration detected in the plasma samples from patients with breast cancer after administration of fucoidan. The amount of fucose obtained after this subtraction was considered as that came from the absorbed fucoidan.

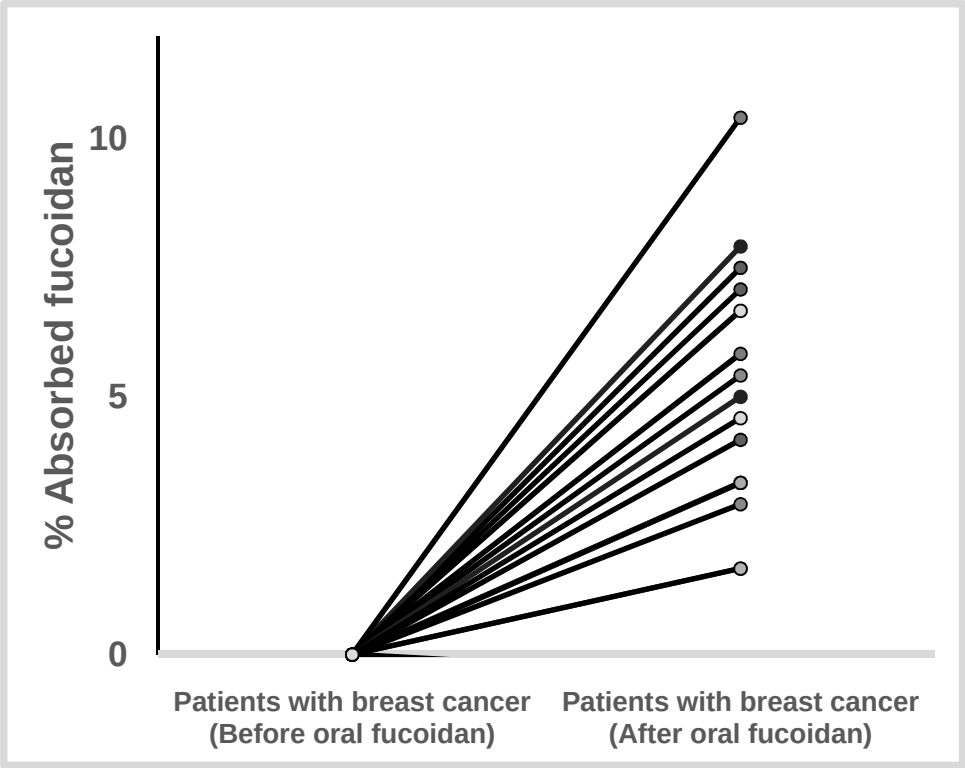

Figure 3.38 The percentage of the absorbed fucoidan in each plasma samples from patients with breast cancer before and after administration of fucoidan

Figure 3.38 shows the percentage of the absorbed fucoidan. The value was obtained based on the amount of fucose in each patient's plasma sample; referring to fucose concentration in fucoidan was 20.35% as described in sub chapter 3.5.2. Figure 3.38 clearly shows that fucoidan

was absorbed following oral administration. Based on the elevated fucose concentration, the total percentage of absorbed fucoidan was 14.28% (n=19). This value was higher compared to that reported in the earlier studies which suggested that fucoidan was absorbed by 0.6% and even lower by 100 to 1000 times as was claimed in another study[63,81]. This suggested that, despite its very polar, big molecule and highly negatively charged structure; to some extent fucoidan can be absorbed following oral administration. Moreover, it can also be concluded that the method developed in this study was proven to be appropriate for the determination of fucoidan in plasma samples.

Chapter 4 - General Conclusion

Fucoidan has been extensively studied for more than a decade for its potential pharmacological activities. It is currently available in the oral dosage form. However, the absorption of fucoidan after oral administration is currently unknown. To our knowledge, there are only two studies that have investigated the amount of absorbed fucoidan after its oral administration. These studies have used an ELISA method to detect the fucoidan in plasma before and after its administration. However, the validity of this method is questionable because of its potential cross-reaction with endogenous polysaccharides such as heparin and related endogenous molecules.

In this study, we developed a new approach for the determination of fucoidan absorption after its oral administration. Considering high polydispersity and molecular weight of fucoidan, analysing fucoidan in its intact form is a laborious and time consuming process. On top of this, it is still not clear whether the fucoidan after its oral administration is absorbed without being structurally converted in the GIT. Therefore, we decided to take an indirect approach to determine the absorption of fucoidan after its oral administration. This indirect approach involved the complete chemical degradation of fucoidan into fucose, its simplest structure-constituting monosaccharide. This increased the specificity of the developed HPLC method since fucose is a single and well characterised molecule. Fucose also has, compared to that of fucoidan, a considerably low molecular weight (164.16 g/mol)[120]. Therefore, it eased its separation and detection in plasma sample.

Among several different chromatographic methods tested in this study, fucose in blank plasma as well as patients' plasma samples was well separated from other endogenous compound using a normal phase chromatography. The mobile phase consisted of 2.5 mM Ammonium acetate

(pH 4.75) and ACN (20:80) with the flow rate of 1.0 mL/minute. The column was a Shodex Asahipak NH2P-50 4E column and was kept in the column compartment under 30 °C. The samples were kept at 10 °C in the sample compartment and was injected in the volume of 10 µL each time. Detection was performed using CAD with the particular set PF of 1.0 and FV of 3. Patients' plasma samples were treated with ACN to precipitate plasma protein and fucoidan. The precipitate containing fucoidan was digested using 20% TFA. The performance of the developed HPLC method was also evaluated which showed acceptable intra- and inter-day accuracy, precision and repeatability. This suggested the suitability of the method to be utilised for the determination of fucoidan in the patients' plasma samples.

Fucose concentration was obtained following determination of fucose in plasma samples from healthy volunteers, patients with breast cancer before administration of fucoidan and after administration of fucoidan; using the developed HPLC method. Fucose concentration in healthy volunteers was observed lower (6.5 mg/100 mL, n=10) compared to that of patients with breast cancer before administration of fucoidan (12.8 mg/100 mL, n=19). Increase in fucose concentration was also observed in patients with breast cancer after fucoidan was given which showed fucose concentration of 13.9 mg/100 mL (n=19). The amount of fucose concentration varied between patients but showed consistent elevation among them. This suggested that, with regards to the increase in the concentration of fucose, fucoidan was absorbed following its oral administration.

Finally, this study has provided a new method and approach for the determination of fucoidan in plasma samples following its oral administration. The method offers simplicity compared to the available HPLC methods. For instance, the methods using fluorescence detector which require derivatisation of fucose to improve separation and detection. It also offers specificity

compared to the available ELISA methods which was suggested to have cross-reaction between the developed antibody and polysaccharides other than fucoidan. Also, the chosen chemical degradation of fucoidan will probably put the method to a lower cost and less time consuming compared to that of enzymatic procedure. The performance of the method has also been tested and showed acceptable result. The application of the method to the patients' plasma samples has also been investigated and it can be concluded that fucoidan was absorbed after its oral administration. Further attempt, however, can be useful in investigating the mechanisms by which fucoidan is absorbed after its oral administration.

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